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## REDACTORES

*Y. REENPÄÄ*  
*Helsinki*

*A. KROGH*  
*Kjöbenhavn*

*E. LANGFELDT*  
*Oslo*

*G. LILJESTRAND* (EDITOR)  
*Stockholm*

## COLLABORANTES

G. AHLÖREN (Lund), Y. AIRILA (Helsinki), E. L. BACKMAN (Uppsala), S. BERGSTRÖM (Lund), C. G. BERNHARD (Stockholm), G. BLIX (Uppsala), J. BOCK (Kjöbenhavn), R. EGE (Kjöbenhavn), H. v. EULER (Stockholm), U. S. v. EULER (Stockholm), A. FÖLLING (Oslo), R. GRANIT (Stockholm), G. GÖTHLIN (Uppsala), E. HAMMARSTEN (Stockholm), E. HANSEN (Kjöbenhavn), E. HORWÜ-CHRISTENSEN (Stockholm), G. KARLSON (Lund), F. LEEGAARD (Oslo), J. LEBMANN (Göteborg), E. LUNDGAARD (Kjöbenhavn), K. MÖLLER (Kjöbenhavn), R. NICOLAYSEN (Oslo), S. ØRSKOV (Århus), A. V. SAHLSTEDT (Stockholm), F. SCHÖNHEYDER (Århus), P. E. SIMOLA (Helsinki), K. SJÖBERG (Stockholm), T. TEORELL (Uppsala), H. THEORELL (Stockholm), T. THUNBERG (Lund), A. WESTERLUND (Uppsala), A. I. VIRTANEN (Helsinki), Y. ZOTTERMAN (Stockholm), G. ÅGREN (Uppsala)

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## The Active Ion Transport through the Isolated Frog Skin in the Light of Tracer Studies.

By

HANS H. USSING.

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In 1937 KROGH showed that frogs in need of salt will take up sodium chloride through the skin even from very dilute solutions. And since then a similar ion uptake has been found to exist in a large number of fresh water animals.

The question remained, however, whether this active uptake is a specific response to need of salt or whether an ion uptake is always going on, the effect of which is concealed by an equal loss due to diffusion. This question cannot be answered by classical chemical methods.

The use of isotopes as tracers makes possible the determination of the "flux", the amount of a given ion species which per unit time crosses unit area of a membrane in one direction. Under certain conditions it may be permissible to identify the inward flux of an ion species with the active uptake across the membrane, namely where it can be assumed that the amount of the ion in question which passes in by diffusion is negligible compared with the total flux.

This approach to the determination of the true rate of active ion uptake in fresh water animals has been used by BARKER JØRGENSEN, LEVI and USSING (1946) for the determination of the sodium uptake in the axolotl and by HOLM-JENSEN (1948) for the determination of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  uptake in *Daphnia magna*. In both cases the isotope method gave reasonable figures for the



active uptake. In the axolotl Na-influx figures were sometimes found which were not more than 50 % in excess over the net salt uptake. However, we observed that injury to the skin of the animals — for instance pricking with a needle — resulted in a short lasting but violent increase in the influx as well as in the outflux of  $\text{Na}^+$ .

The tentative explanation of the phenomenon given in the paper cited was that the pricking might stimulate the salt uptake as well as the salt loss via different hormones. It was, however, equally possible that the stimulation of the skin brings about a condition in the skin, allowing a simple exchange of sodium ions comparable with the exchange going on between the surface layer of a crystal and the surrounding solution. It might be of interest therefore to consider the conditions which will allow such an exchange without consumption of metabolic energy, the more so because such a condition for Na-exchange seems to exist in the cell membrane of striated muscle fibers (USSING 1947, LEVI and USSING, 1948).

In the first place such an exchange of an ion species between two phases may take place if the difference in activity of the ion in the two phases is balanced by a corresponding difference in electric potential. A typical example is the Donnan equilibrium; but quite generally we have for diffusible ions that they are in equilibrium when

$$\psi = \frac{R \cdot T}{n F} \cdot \ln \frac{[a_i]}{[a_o]}$$

where  $\psi$  is the membrane potential,  $R$  the gas constant,  $T$  the absolute temperature,  $n$  the valency of the ion,  $F$  Faradays number and  $a_i$  and  $a_o$  the activities of the ion in the inside and outside solutions respectively. It is well known that as a rule the inside of the frog skin is positive relative to the outside, when the inside is bathed with Ringer and the outside with dilute NaCl-solution. A few observations indicate that the same is the case in the axolotl so that it is doubtful whether this equilibrium exchange is of importance for the sodium ion. An equilibrium exchange of chloride ions is on the other hand quite likely.

There are, however, certain mechanisms which will allow an exchange of an ion species without consumption of energy even between phases where the difference in concentration is not balanced by electrical forces. Such a system can be realized in

different ways (compare Ussing l. c.). Such a hypothetical exchange system can be simply visualized as a Na-impermeable mono layer which contains scattered anions ( $B^-$ ) of a substance which forms a stable complex with Na. Due to the thermal movements these complex molecules will sometimes come into contact with the outside medium and sometimes with the inside medium. If the inside solution contains  $Na^{21}$  ions these may exchange with  $Na^{22}$  in some of the complex molecules; and when later these molecules touch the outside solution  $Na^{21}$  will leave the complex in exchange for  $Na^{22}$  which is present here. In its ideal form such a mechanism will always take one Na ion when it gives another so that no net change in the Na-concentrations need take place although there may be an appreciable flux in both directions.

In the foregoing we have assumed that  $B^-$  would only form a complex with  $Na^+$ . In fact  $B^-$  will form at least one uncharged complex more, namely the corresponding acid  $BH$ , and therefore the foregoing considerations are only strictly valid in a region where the Na-concentration is very high compared with the  $H^+$  ion concentration.

Generally we have, however:

$$\frac{[BH]}{[B^-]} \cdot K_H = [H^+] \text{ and } \frac{[BNa]}{[B^-]} \cdot K_{Na} = [Na^+]$$

(the figures in brackets denote thermodynamic activities).  $K_H$  is the acid dissociation constant and  $K_{Na}$  the instability constant of the complex  $BNa$ . Combining these equations we get:

$$\frac{[BNa]}{[BH]} = \frac{[Na^+]}{[H^+]} \cdot \frac{K_H}{K_{Na}} = \frac{[Na^+]}{[H^+]} \cdot K$$

This means that if in the two phases pH is the same, more  $BH$  will be formed in the phase where  $[Na^+]$  is lowest. This means that more  $BH$  will go from the o-system to the i-system than in the opposite direction, whereas the reverse is the case with  $BNa$ . Thus  $Na^+$  will to some extent exchange with  $H^+$ . What will happen further depends on the properties of the membrane. If it is impermeable to anions the exchange of  $H^+$  against  $Na^+$  will continue, increasing pH of the o-system and lowering pH of the i-system until  $[H^+]_o \cdot [B^+]_o = [H^+]_i [B^-]_i$ . Then the Na-flux will again be the same in both directions.

If, on the other hand, the membrane is permeable to anions the exchange of  $H^+$  with  $Na^+$  will lead to a constant leakage of salt

from  $i$  to  $o$  because  $\text{Cl}^-$  ions may go through in exchange for  $\text{OH}^-$ , which in turn combine with the  $\text{H}^+$  ions carried to  $i$  in exchange for  $\text{Na}^+$ . But even if such leakage is going on, we may find a proportion between  $\text{Na}$ -influx and  $\text{Na}$ -outflux near unity under conditions where the assumption of a diffusion of free ions would lead to a very small figure.

These considerations show, that whatever concentration difference and whatever potential difference is found between two phases we cannot beforehand be certain that the flux of an ion species is a true measure of the active uptake. We have found (USSING 1947, LEVI and USSING 1948) that through the membrane of the striated muscle fibre the  $\text{Na}^+$  outflux is quite likely considerably higher than the active  $\text{Na}^+$  extrusion.

Below, it will be shown that it becomes a matter of definition whether the  $\text{Na}^+$  influx through the frog skin should be considered identical with the active uptake.

For the experiments presented here the isolated skin from the abdomen of the frog (*Rana temporaria*) was used. With its folds and wrinkles, the skin of the axolotl is not so well suited as a membrane as is the frog skin. Moreover it is of great value in the discussion of the results to make use of the large amount of knowledge already present concerning the variations of the potential difference across the frog skin under different conditions. Especially the very interesting potentiometric analysis of the skin structure performed by MEYER and BERNFELD (1946) has been of importance for the present study, although the interpretation which these authors give to their results cannot be entirely correct. They regard the frog skin as consisting of at least four layers of different permeability, two of which are more closely studied, namely the outer epithelial layer showing cation selectivity and a deeper layer which is specifically permeable to  $\text{H}^+$  ions. The major part of the resting potential is regarded as being due to the "difference in  $\text{H}^+$  concentration on both sides of the last mentioned membrane". The evidence given for the  $\text{H}^+$  ion selectivity of the layer mentioned looks convincing but nevertheless the tracer experiments show that  $\text{Na}^+$  and  $\text{Cl}^-$  in considerable amounts pass through. MEYER and BERNFELD in their discussion pay no attention to the fact first stated by HUF (1935) that the isolated frog skin is able to transport salt from the outside to the inside, when both sides are bathed with Ringer, and that this transport is closely related to the potential difference measured.

With the tracer technique the active uptake can also be ascertained when the outside concentration of NaCl is lower than in Ringer and, moreover, the  $\text{Na}^+$  ion and the  $\text{Cl}^-$  ion can be studied separately.

The purpose of the present study has been to measure this "flux" in both directions of sodium and chloride ions through the isolated frog skin, as a function of a number of factors, namely outside salt concentration and outside and inside pH under fairly normal conditions (ample  $\text{O}_2$  supply, Ringer solution on inside of the skin). Further studies have been concerned with the effect of adrenaline and of cyanide poisoning on the flux values. Adrenaline has been found (BARKER JØRGENSEN, 1947) to increase enormously the permeability of frog skin to salt. Cyanide according to HUF (1935) blocks the active salt transport through the skin.

Finally the potential difference across the skin has been measured as a function of the factors studied.

Based on this material a hypothesis is put forward which will account for the interrelations found between the in- and outflux of ions, the potential difference, and the ion transport.

### Technique.

The apparatus used is shown in fig. 1. A suitable piece (ca  $3 \times 3$  cm) of abdominal skin of a female frog is placed as a membrane between the ground edges of two cylindrical celluloid cups. Each cup fits in a flat depression in an ebonite plate, and these plates can be pressed together between two centered screws.

The solutions in the two chambers thus formed on either side of the skin are circulated by blowing air or any gas mixture wanted through the side tube *A*. The solutions will then ascend to the funnels *F* through the tubes *B* and return to the chambers through the tubes *C*. From the funnels *F* samples can be drawn. If the total contents of for instance the inside circulation system is to be removed, an arterial clamp is placed on the rubber tube *D*. Then the pressure air cannot escape that way and will force the solution in the chamber up into the funnel. From here the solution is removed with a pipette or with the suction pump. The funnels are further used for making contacts between the solutions and two calomel electrodes *E*. The contacts are made through the capillary tubes *G*. One end dips in the funnel and the other end in a small tube *H* filled with saturated KCl solution. Before a measurement of the potential difference is made, *H* is lowered and the solution is allowed for a moment to flow through *G* before *H* is elevated again just so much that a little KCl solution penetrates into the end of the capillary. In this way a fresh contact can always



measured into an aluminium counting dish with a Krogh syringe pipette or a Carlsberg semiautomatic pipette. To small samples distilled water was added to make at least 200  $\mu$ l. Then a drop of concentrated glucose solution was added and the dish heated in an electric oven to ca. 100° to evaporate the water. The sugar on heating will caramelize and cover the bottom of the dish with a sticky layer hindering the creeping of the salt during the crystallisation. In this way the active sodium will be very evenly distributed on the bottom of the dish. If  $\text{Cl}^{35}$  was present the counting of  $\text{Na}^{24}$  was postponed until the short lived  $\text{Cl}^-$  had decayed.

$\text{Cl}^{35}$  was brought to counting as  $\text{AgCl}$ . In an angle centrifuge tube the sample was mixed with sufficient inactive  $\text{NaCl}$  solution to bring the total amount of  $\text{Cl}^-$  to 5  $\mu$  eqv. Water was added to make ca. 5 ml and  $\text{AgCl}$  precipitated with 5 ml of 0.2 n  $\text{AgNO}_3$  in 65 %  $\text{HNO}_3$ . The sample was centrifuged and the precipitate was washed thrice with 10 ml water. As well known  $\text{AgCl}$  forms masses which cling persistently to glass. In order to transfer the samples quantitatively to the counting dishes, the  $\text{AgCl}$  precipitate was therefore suspended in glycerine and the mixture agitated with a glass pin. In glycerine  $\text{AgCl}$  forms a very even suspension which can be transferred quantitatively to the counting dish. The dishes were of the type with a number of perforations in the bottom, thus enabling their use as Büchner funnels. The  $\text{AgCl}$  suspension was filtered on such a funnel using moderately strong suction. The transfer of precipitate was made practically quantitative through one washing with glycerine and two with alcohol. The alcohol washings served further to remove glycerine from the precipitate, so that after a short drying only  $\text{AgCl}$  was left on the filter paper in the dish. Another sheet of filter paper was placed on the top of the precipitate and then this was pressed to a solid cake with a steel piston fitting into the dish.

The short half life of  $\text{Cl}^{35}$  sets rather narrow limits for the extension of the experiments with this isotope. It can easily be shown that when the flux of a radioactive isotope through a membrane is to be measured there is an optimum for the duration of the experimental periods.

If back diffusion of activity can be neglected, the influx of active ions must be proportional to time; however, the active ions which have passed through will decay according to  $y_t = y_0 \cdot e^{-\lambda t}$ . The amount of activity which can be found in the phase originally free from activity is therefore  $y = K \cdot t \cdot e^{-\lambda t}$ , where  $y$  is the activity (for instance in kicks per ml and per minute),  $t$  the time and  $\lambda$  the mean life of the isotope; by differentiation we find

$$\frac{dy}{dt} = k [-\lambda t \cdot e^{-\lambda t} + e^{-\lambda t}]$$

$y$  is maximum for  $\frac{dy}{dt} = 0$  or  $t = \frac{1}{\lambda}$ . In the case of  $\text{Cl}^{35}$  this means that the experimental period should be  $\frac{1}{1.06} = 57$  minutes if maximum accuracy is wanted.

The following example may serve to illustrate the general procedure of the experiments.

12<sup>15</sup> Frog killed, abdominal skin excised and stored in vessel with Ringer solution.

12<sup>30</sup> Skin placed in apparatus. In outside compartment 25 ml distilled water, in inside compartment 25 ml Ringer. Atmospheric air used for mixing.

13<sup>10</sup> 1½ ml active NaCl solution (not made up to Ringer) added to outside solution.

13<sup>20</sup> Samples from inside  $\left\{ \begin{array}{l} 2 \times 1 \text{ ml for Cl-counting} \\ 2 \times 0.5 \text{ ml for Na-counting} \end{array} \right.$   
 Samples from outside  $\left\{ \begin{array}{l} 2 \times 1 \text{ ml for Cl-counting} \\ 2 \times 1 \text{ ml for Cl-titration} \end{array} \right.$   
 14<sup>20</sup> Samples from inside  $\left\{ \begin{array}{l} 2 \times 5 \text{ ml for Cl-counting} \\ 2 \times 0.1 \text{ ml for Na-counting.} \end{array} \right.$

Inside solution sucked out and used for pH determination. 25 ml fresh Ringer poured in instead.

14<sup>35</sup> Samples from inside  $\left\{ \begin{array}{l} 2 \times 1.5 \text{ ml for Cl-counting} \\ 2 \times 0.1 \text{ ml for Na-counting.} \end{array} \right.$

45 µl 1 ‰ adrenalin hydrochloride added to inside solution.

14<sup>45</sup> Samples from outside: 1 ml for Cl-titration.

15<sup>35</sup> Samples from inside  $\left\{ \begin{array}{l} 2 \times 5 \text{ ml for Cl-counting} \\ 2 \times 0.1 \text{ ml for Na-counting.} \end{array} \right.$

Inside solution sucked out and used for pH-determination. Inside compartment washed three times with Ringer and filled with 25 ml fresh Ringer.

15<sup>50</sup> Samples from inside  $\left\{ \begin{array}{l} 2 \times 2 \text{ ml for Cl-counting} \\ 2 \times 0.1 \text{ ml for Na-counting.} \end{array} \right.$

16<sup>00</sup> Sample from outside: 1 ml for Cl-titration.

16<sup>50</sup> Samples from inside  $\left\{ \begin{array}{l} 2 \times 5 \text{ ml for Cl-counting} \\ 2 \times 0.1 \text{ ml for Na-counting.} \end{array} \right.$

Rest of outside and inside solutions sucked out and used for pH-determination, Na-analysis and Na-counting.

Readings of the potential difference were made at intervals all during the experiment. The countings and analysis from this experiment are given in table I.

Chloride titrations were performed according to SCHNOHR (1934). For sodium estimations the method of HOFFMAN and OSGOD (1938) was used in the modification described by BARKER JØRGENSEN, LEVI and USSING (l. c.).

#### Calculations:

In most cases less than 1 % of the activity added in one of the compartments passes on to the other within the experimental period so that the activity can be regarded as constant on the side where addition has taken place. The total salt concentrations as a rule can be regarded as constant too (exceptions: adrenaline stimulation and cyanide poisoning). This means that for example the influx of sodium ion in an experiment where Na<sup>24</sup> was added to the outside solution can be calculated as follows.

Table I.

		Cl <sup>-</sup> conc. mmol	Na- conc. mmol	Na <sup>24</sup> activity counts/ min. ml corrected for decay. Zero time 10 <sup>00</sup>	Cl <sup>36</sup> activity counts/ min. ml corrected for decay. Zero time 18 <sup>00</sup>	Circu- lating volume, ml	pH	P.D. mV (inside posi- tive)
1' period	13 <sup>30</sup> { Outside	3.47	3.47	210 × 10 <sup>3</sup>	12,600	26.5	6.8	+85
	13 <sup>30</sup> { Inside	115	115	101.5	1.83	25	—	
	14 <sup>30</sup> { Outside	—	—	—	—	22.5	—	
	14 <sup>30</sup> { Inside	—	—	1,800	32	22	8.22	
2' period	14 <sup>35</sup> { Outside	3.47	—	—	—	22.5	—	+33
	14 <sup>35</sup> { Inside	—	—	470	10.7	26	—	
	15 <sup>05</sup> { Outside	—	—	—	—	22.5	—	
	15 <sup>05</sup> { Inside	—	—	3,640	40	22.8	7.97	
3' period	15 <sup>20</sup> { Outside	3.62	—	—	—	22.5	—	+67
	15 <sup>20</sup> { Inside	—	—	677	6.25	26	—	
	16 <sup>30</sup> { Outside	3.70	3.7	200 × 10 <sup>3</sup>	12,600	22.5	6.82	
	16 <sup>30</sup> { Inside	—	—	3,045	50	21.8	7.97	

If  $M_{Na(o)}$  is the total amount of  $Na^+$  in the outside solution,  $\Delta Na^{24}(i)$  the increase in one hour in  $Na^{24}$  counts in the total inside solution and  $Na^{24}(o)$  the number of counts in the total outside solution (all activities corrected for decay) and  $A$  is the exposed area of the frog skin the flux  $F$  is determined by

$$F = \frac{M_{Na(o)} \cdot \Delta Na^{24}(i)}{A \cdot Na^{24}(o)}$$

The flux is given as  $\mu$  equivalents/cm<sup>2</sup>, hour. If the outside solution shows appreciable variations in concentration and activity it may be preferable to use the formulae derived in the paper of BARKER JØRGENSEN, LEVI and USSING (l. c.).

## Results.

### 1. The Influence of Outside $Na^+$ -concentration on $Na^+$ -influx.

The influence of stepwise increase of the outside NaCl-concentration on the  $Na^+$ -influx is shown in fig. 2. The active NaCl was added to the outside solution and with two hours interval inactive NaCl in substance was added. The flux measured in the second hour after each change in concentration was used for drawing the curves, which represent two separate experiments. It is seen that



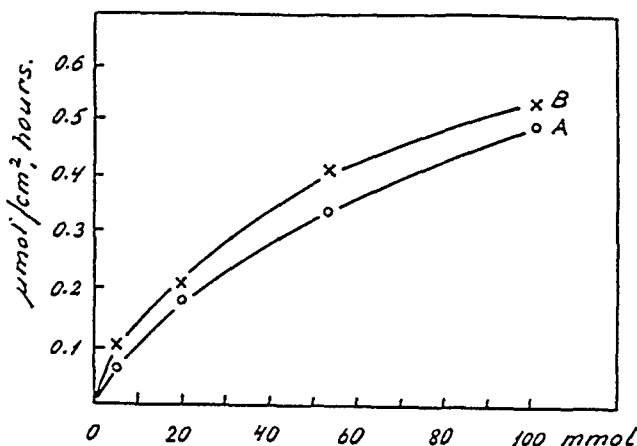


Fig. 2. The  $\text{Na}^+$ -influx through the isolated frog skin as a function of outside  $\text{NaCl}$ -concentration. The  $\text{NaCl}$ -concentration is raised in steps by addition of solid  $\text{NaCl}$ . A and B denote two separate experiments.

there is no proportionality between outside concentration and  $\text{Na}$ -influx; when the outside concentration increases the relative increase in flux is diminished. It cannot be decided whether the flux approaches an asymptote or not.  $\text{pH}$  in outside solutions was about 5.5 in experiment A and about 6.1 in experiment B. Inside  $\text{pH}$  in both experiments 8.3 (this reaction is brought about by using ordinary Ringer and mixing with atmospheric air).

In reality it cannot be decided from these experiments whether the increase in outside concentration is the only reason for the change in  $\text{Na}$ -influx. The total experiment lasting for about 8 hours, there might be some influence of the ageing of the skin. In fig. 3, however, we have plotted the influx of  $\text{Na}^+$  as a function of outside concentration using data from 20 different experiments. In all cases the inside  $\text{pH}$  was 8.3 and outside  $\text{pH}$  near the neutral point (as will be shown later the inside  $\text{pH}$  exerts a very pronounced influence on the influx of  $\text{Na}^+$ , whereas outside  $\text{pH}$  seems of no decisive influence between 5 and 8). The figure shows a considerable scattering of the observations but the general trend is obviously the same as in fig. 2.

## 2. The Influence of Outside $\text{Na}$ -concentration on $\text{Na}^+$ -outflux.

Fig. 3 also shows all the observations made concerning outflux of sodium ( $\text{Na}^{24}$  in these experiments added on inside). The observations were made under conditions as far as possible identical with those prevailing under the influx determinations. The out-



Table II.

*The influence of outside salt concentration on the Na<sup>+</sup>-outflux through the isolated frog skin.*

Period number (one hour each)	Outside NaCl-conc. mmol.	Na <sup>+</sup> -outflux μmol/cm <sup>2</sup> , hour.
1'	115	0.132
2'	115	0.151
3'	1	0.154
4'	1	0.045
5'	1	0.040

dependance on the outside concentration. Especially at very low outside concentrations the outflux goes down. This is clearly seen for instance from table II. Here the Na<sup>+</sup>-outflux was measured with Ringer outside for two hours; then the outside concentration was lowered to one millimolar NaCl. In the first one hour period after the change of concentration the outflux remained high, but then it dropped to a new level about one third of the former one.

This time lag between change in concentration and change in outflux is always found and may be ascribed to some change in the structure in the surface of the skin, perhaps comparable with the precipitation of globulines in distilled water. Whatever the reason, this tightening of the skin at low concentrations may be very useful for the animals because it will lower the Na<sup>+</sup> loss in waters where the salt content is too low to enable an efficient up-take.

### 3. The Influence of pH on the Na<sup>+</sup>-influx.

pH changes were brought about either by addition of phosphate buffers to the solutions or by using gas mixtures with different CO<sub>2</sub>-content for circulating the solutions. Whereas phosphate buffers will only slowly penetrate into the transporting cells, a change in CO<sub>2</sub>-tension must be assumed very soon to exert an effect also on the pH of the protoplasm of the cells.

#### a. Influence of CO<sub>2</sub>-tension.

Table III shows a series of experiments where the CO<sub>2</sub>-tension in the whole system was changed from 12.4 mm Hg (1½ % CO<sub>2</sub>) to that prevailing in atmospheric air, and back again.

The influx of Na<sup>+</sup> and Cl<sup>-</sup> was determined in one hour periods. Between the measuring periods intervals lasting 15 minutes were interposed allowing the gas mixture to equilibrate with the solu-

Table III.

*Influence of pH on the influx of sodium and chloride ions through the isolated frog skin. Each experiment comprises three successive one hour periods. The pH changes are brought about by changing the CO<sub>2</sub>-tension of the whole system.*

	Concentration mmol		pH		Na passing in "mol/hour, cm <sup>2</sup>	Cl passing in "mol/hour, cm <sup>2</sup>
	inside	outside	inside	outside		
I	115	2.87	7.1	5.8	0.022	0.012
	"	"	8.3	7.1	0.073	0.014
	"	"	7.1	5.8	0.027	0.009
II	115	8.04	7.1	5.7	0.030	0.031
	"	"	8.3	6.9	0.098	0.116
	"	"	7.1	5.7	0.023	0.008
III	115	3.32	7.2	5.7	0.025	0.006
	"	"	8.3	7.1	0.097	0.012
	"	"	7.2	5.7	0.064	0.011
IV	115	10.00	7.2	5.45	0.059	0.007
	"	"	8.3	6.87	0.170	0.025
	"	"	7.2	5.45	0.094	0.013

tions. It is seen that the Na<sup>+</sup>-influx is about 3 times higher at the low CO<sub>2</sub>-tension than at the high one. The relative change in Na<sup>+</sup>-influx following a shift of CO<sub>2</sub>-tension is but little dependent on the salt concentration in the outside solution. This is born out in table IV which shows the proportions found in a series of experiments between the Na<sup>+</sup>-influx during air mixing and during 1½% CO<sub>2</sub>-mixing; the experiments are arranged according to increasing

Table IV.

*The proportions found for a number of frog skins between the Na<sup>+</sup>-influx measured during air mixing and during 1½% CO<sub>2</sub> mixing. The experiments are arranged according to increasing outside NaCl-concentration.*

Outside NaCl-conc. mmol	Na <sup>+</sup> -influx (air)/Na <sup>+</sup> -influx (1½% CO <sub>2</sub> )
2.87	3.32
3.32	3.88
5.2	2.39
8.04	3.26
10.0	2.88
58	1.88
115	2.75
115	3.32

Table V.

*The influence of inside pH on the Na<sup>+</sup>-influx. The pH changes are brought about by the addition of phosphate buffers.*

	Period number (one hour each)	NaCl-conc. (mmol)		pH		Na <sup>+</sup> -influx $\mu\text{mol}/\text{cm}^2$ , hour	P. D. mV sign refers to inside
		inside	outside	inside	outside		
I	1' .....	115	1.65	6.72	6.5	0.018	— 8
	2' .....	"	"	"	"	0.010	—
	3' .....	"	"	7.77	"	0.060	+14
	4' .....	"	"	"	"	0.061	+19.5
II	1' .....	115	1.64	7.77	6.5	0.095	—
	2' .....	"	"	"	"	0.050	—
	3' .....	"	"	6.72	"	0.039	—
	4' .....	"	"	"	"	0.015	—
III	1' .....	115	6.60	8.1	6.3	0.318	+82
	2' .....	"	"	"	"	0.276	—
	3' .....	"	"	7.2	"	0.161	+51
	4' .....	"	"	"	"	0.070	+38

outside salt concentration. The constancy of the proportion must be taken to mean that the influx of Na<sup>+</sup> shows the same type of dependence on outside salt concentration at these two CO<sub>2</sub>-tensions.

b. Na<sup>+</sup>-influx as a function of inside pH.

As shown in table V the Na<sup>+</sup>-influx is much higher when the inside pH is about 8 than when the reaction is about neutral. However, it is apparent from the table that the adjustment of the flux to a new pH value requires some time. Whereas the adjustment of the flux after a shift in CO<sub>2</sub>-tension takes place in less than a quarter of an hour the flux is still changing after one hour when phosphate buffer has been used for shifting pH.

Moreover the changes due to pH alterations seem to be superimposed on a steady fall with time of the Na<sup>+</sup>-influx. Such a tendency was not apparent in the experiments with phosphate-free Ringer (compare table III); in long lasting experiments it may, however, be seen.

c. Na<sup>+</sup>-influx as a function of outside pH.

Table VI shows an experiment where the outside pH was changed in steps by addition of dilute phosphoric acid, whereas the inside pH was kept constant at 8.0. The Na<sup>+</sup>-influx was but little influenced by the shifting of pH from 6.6 to 5.25. When, however,

Table VI.

The influence of outside pH on the influx of  $\text{Na}^+$  and  $\text{Cl}^-$ . The outside pH is lowered in steps by the addition of phosphoric acid.

Period number (one hour each)	NaCl-conc. mmol		pH		Na <sup>+</sup> -influx $\mu\text{mol}/\text{cm}^2$ , hour	Cl <sup>-</sup> -influx $\mu\text{mol}/\text{cm}^2$ , hour
	inside	outside	inside	outside		
1' .....	115	6.36	8.0	6.6	0.105	0.083
2' .....	115	6.36	8.0	5.25	0.097	0.055
3' .....	115	5.44	8.0	3.3	0.012	0.175

Table VII.

The influence of outside pH on the influx of  $\text{Na}^+$ . The outside pH first fixed at 3.3 by the addition of phosphoric acid; later the pH was raised to 4.65 by addition of a minute amount of secondary phosphate.

Period number (one hour each)	NaCl-conc. mmol		pH		Na <sup>+</sup> -influx $\mu\text{mol}/\text{cm}^2$ , hour
	inside	outside	inside	outside	
1' .....	115	6.4	8.3	3.3	0.008
2' .....	"	"	"	4.65	0.016
3' .....	"	"	"	4.65	0.015

the outside pH was lowered to 3.3 the  $\text{Na}^+$ -influx nearly stopped. Table VII shows a similar experiment, only that pH was lowest in the first period (3.3) and was then shifted to 4.65, again it is seen that the Na-influx is extremely low at pH 3.3 and even the value found at 4.65, namely  $0.015 \mu\text{mol}/\text{cm}^2$  hour is very low compared with what is normally found with an outside NaCl concentration of 6.3 millimoles and an inside pH of 8.3 (see fig. 3).

Thus it appears that at an outside pH somewhere between 5.25 and 4.65 the  $\text{Na}^+$ -influx begins to fall off rapidly. At the same time the  $\text{Cl}^-$ -influx goes up (table VI).

This observation does probably mean that the pace setting factor for the  $\text{Na}^+$ -influx at low pH is the permeability of the surface layer of the outside, which according to AMBERSON (1936) has its isoelectric point at 5.1. At higher pH the surface layer is predominantly permeable to cations and at lower pH to anions.

#### 4. The Influence of $\text{CO}_2$ -tension on the Outflux of $\text{Na}^+$ .

Only in one experiment was the influence of the  $\text{CO}_2$ -tension on  $\text{Na}^+$ -outflux studied. No significant difference was found whether the  $\text{CO}_2$ -tension was high or low.

### 5. $\text{Na}^+$ -influx and Potential Difference (P.D.).

The  $\text{Na}^+$ -influx and the P.D. seem to be very intimately inter-related. According to the observations of MEYER and BERNFELD (l. c.) the P.D. changes in a characteristic fashion with the outside NaCl-concentration, so that higher NaCl-concentrations make the inside increasingly positive relative to the outside. The change in P.D. takes place in a way which recalls of the way the  $\text{Na}^+$ -influx changes with outside concentration (compare fig. 2).

MEYER and BERNFELD further found that the P.D. was closely correlated with the inside pH, so that for instance an increase of one pH unit made the inside solution about 58 mV more positive relative to the outside (compare fig. 11 in this paper); the increase in P.D. is observed promptly when the change is brought about by changing the  $\text{CO}_2$ -tension, whereas phosphate buffers bring about the P.D. change only after some time of latency. As we have seen the  $\text{Na}^+$ -influx behaves, qualitatively, in much the same way as the P.D.: increasing when the inside pH increases and performing this increase much more readily when the pH shift is due to a change in  $\text{CO}_2$ -tension than when phosphate buffers bring about the shift.

The rather paradoxical situation is that all factors which make the inside solution more positive relative to the outside will increase the influx of the positively charged sodium ions.

Some examples of the close relationship between  $\text{Na}^+$ -influx and P.D. are given in table V. More examples will be given later.

### 6. The Influx of Chloride Ions.

The most outstanding fact about the  $\text{Cl}^-$ -influx is that it is nearly always considerably lower than the corresponding value for  $\text{Na}^+$ -influx (compare for instance table III). From 24 simultaneous observations of  $\text{Na}^+$ - and  $\text{Cl}^-$ -influx, the  $\text{Cl}^-$ -influx was much lower in 20 cases, about equal in two cases and higher in two cases.

From this it will appear that there is no constant relation between the influx values for these two ions.

The material is too small to allow a description of the relation between outside salt concentration and  $\text{Cl}^-$ -influx. As far as the evidence goes, the influx does, however, increase considerably with increasing outside concentration. From experiments with

pH 8.3 on the inside and about neutral reaction on the outside we find a  $\text{Cl}^-$ -influx about  $0.012 \mu \text{ mol/cm}^2$ , hour at an outside  $\text{NaCl}$ -concentration about 3 mmol.  $\text{NaCl}$  (see table III), whereas it is known from the experiments of HUF (l. c.) and BARKER JØRGENSEN (personal information) that the *net* amount of  $\text{Cl}^-$  transported in by isolated frog skin when bathed with Ringer on both sides is about  $0.40 \mu \text{ mol/cm}^2$ , hour.

The pH of the system as determined by the  $\text{CO}_2$ -tension influences the  $\text{Cl}^-$ -influx in much the same way as this factor influenced the  $\text{Na}^+$ -influx: For the same skin the  $\text{Cl}^-$ -influx is always higher at the higher pH (see table III).

As to the influence of outside pH, we have already seen (p. 15) that the  $\text{Cl}^-$ -influx goes up and the  $\text{Na}^+$ -influx goes down when the outside pH is lowered to 3.3.

As we have seen, an increase in outside  $\text{NaCl}$ -concentration or in inside pH will give a higher  $\text{Cl}^-$ -influx. This again means that if we compare with the P.D. more  $\text{Cl}^-$  will go in the more positive the inside is relative to the outside solution.

#### 7. The Influence of Adrenaline on the Influx and Outflux of $\text{Na}^+$ and $\text{Cl}^-$ .

BARKER JØRGENSEN (l. c.) has shown the isolated frog skin to react in a very peculiar way on the addition of adrenaline to the inside solution (added on the outside adrenaline is without effect): When the inside of the skin is bathed with Ringer and the outside with distilled water a very slow leakage of salt is found. Immediately after the addition of one part in a million of adrenaline hydrochloride to the inside solution, salt begins to go through some 20 times as fast as before and will continue to do so until the adrenaline is removed.

Now it would be of interest to see how adrenaline would influence the influx of ions. It turned out that adrenaline (1 to  $10^{-6}$ ) increased the influx of sodium considerably; in most cases the  $\text{Na}^+$ -influx was about doubled (see tables VIII and IX) irrespective of the outside salt concentration. The influx of  $\text{Cl}^-$  seemed to be much less affected. From the data hitherto collected it cannot be decided whether the  $\text{Cl}^-$ -influx is at all influenced by adrenaline.

At the same time the outflux of  $\text{Cl}^-$  as well as of  $\text{Na}^+$  was very much increased. This can be inferred from the increase in outside



Table VIII.

*Influence of adrenaline on the  $\text{Na}^+$ -influx and  $\text{Cl}^-$ -influx through the isolated frog skin. Each experiment comprises three successive one hour periods.*

	NaCl mean concentration mmol		Adrenaline conc. on inside	$\text{Na}^+$ -influx $\mu\text{mol}/\text{cm}^2$ , hour	$\text{Cl}^-$ -influx $\mu\text{mol}/\text{cm}^2$ , hour
	inside	outside			
I	115	3.47	0	0.092	0.028
	,	3.55	$1/10^6$	0.185	0.031
	,	3.66	0	0.141	0.042
II	115	9.08	0	0.149	0.044
	,	9.16	$1/10^6$	0.305	0.064
	,	9.32	0	0.229	0.096

Table IX.

*The influence of adrenaline ( $1/10^6$ ) on the  $\text{Na}^+$ -influx through the isolated frog skin with Ringer on both sides.*

Period number (one hour each)	NaCl-conc. mmol		Adrenaline conc. on inside	$\text{Na}^+$ -influx $\mu\text{mol}/\text{cm}^2$ , hour
	inside	outside		
1' .....	115	115	0	0.441
2' .....	,	,	,	0.550
3' .....	,	,	$1/10^6$	0.874
4' .....	,	,	,	0.912
5' .....	,	,	,	0.657

Table X.

*The influence of adrenaline ( $1/10^6$ ) on the  $\text{Na}^+$ -outflux through the isolated frog skin with Ringer on both sides.*

Period number (one hour each)	NaCl-conc. mmol		Adrenaline conc. on inside	$\text{Na}^+$ -outflux $\mu\text{mol}/\text{cm}^2$ , hour
	inside	outside		
1' .....	115	115	0	0.098
2' .....	,	,	,	0.097
3' .....	,	,	$1/10^6$	1.820
4' .....	,	,	,	0.985
5' .....	,	,	,	0.740

salt concentration in cases where a concentration gradient exists from the inside to the outside. Even when both sides of the skin are in contact with Ringer, the violent increase in outflux can be demonstrated with the tracer method, see table X.

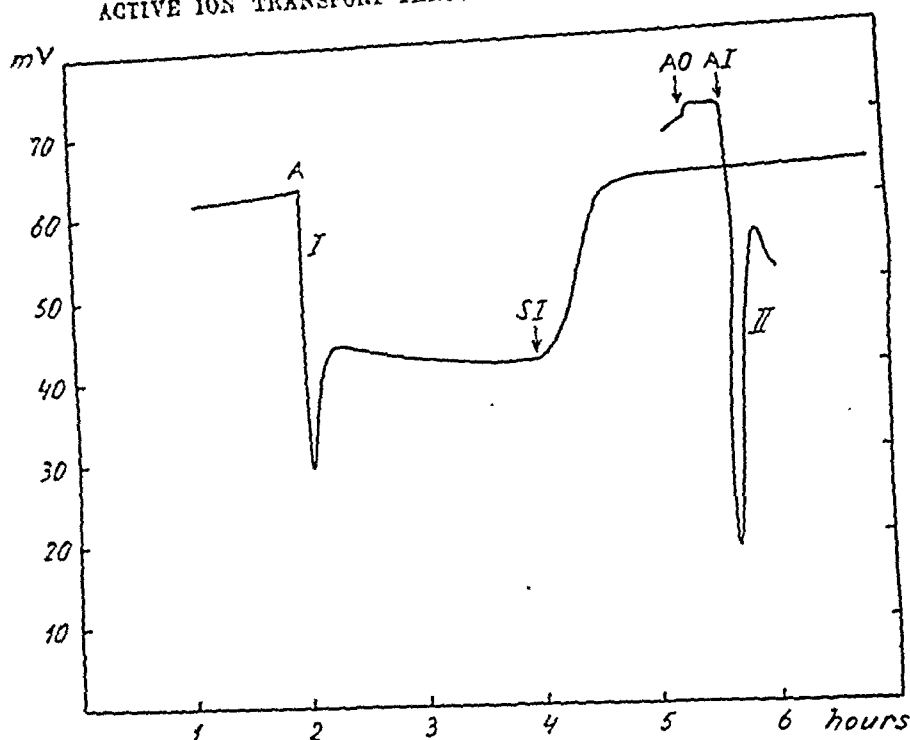


Fig. 4. The influence of adrenaline on the potential-difference (P.D.) across the frog skin (sign related to inside).

Curve I: inside Ringer, outside 1 mmol NaCl.

Curve II: " " " 3.5 " "

At A and AI addition of 46  $\mu$ l 1 ‰ adrenaline to inside solution.

At AO " " " " " outside "

At SI renewal of inside solution.

In fact, after adrenaline the influx and the outflux of  $\text{Na}^+$  are of the same order of magnitude, when Ringer is present on both sides.

#### 8. The Influence of Adrenaline on the P.D. across the Skin.

The addition of adrenaline to the inside solution will always result in characteristic changes in P.D. The typical reaction consists in a sudden fall (the P.D. is considered positive when the inside solution is positive relative to the outside), followed after less than one minute by an increase; later as a rule the P.D. falls again more slowly.

The shape of the P.D. curve depends primarily on the outside salt concentration. When the outside concentration is low the dominating feature after adrenaline is the fall in P.D.; see fig. 4, I; the outside salt concentration is here 1 mmole. The secondary rise does not bring the P.D. back to the initial value.

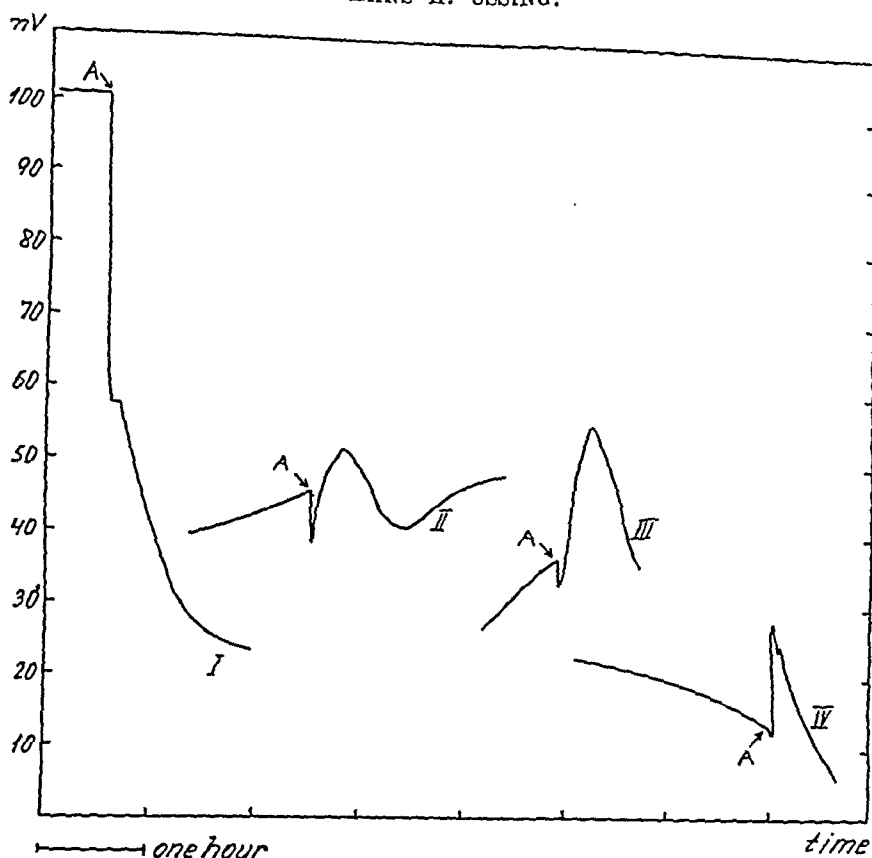


Fig. 5. The influence of adrenaline on the potential difference (P.D.) across the frog skin (sign related to inside). Ringer solution on both sides of the skin. At A addition of 46  $\mu$ l 1  $\%$  adrenaline to inside solution.

A similar case is shown in fig. 4, II; here the outside concentration is 3.5 mmoles. Adrenaline is added first on the outside (without effect) at AO and on the inside at AI.

If on the other hand the outside solution is Ringer, the course of the adrenaline reaction is different. It now apparently depends on whether the P.D. is high or low before the stimulation. If high (about 100 mV) before adrenaline addition the P.D. shows a violent fall with only a faint indication of the secondary rise (fig. 5, I). If the P.D. is relatively low beforehand (lower than 50 mV) adrenaline will only induce a small fall in P.D. and the secondary rise will bring the P.D. well above the starting value before it begins to fall again (fig. 5, II, III, IV).

These P.D. changes induced by adrenaline should probably be explained in the following way:

The initial fall is due to the skin becoming suddenly much more

permeable to  $\text{Na}^+$ -ions. The outside superficial layer being cation-selective (MEYER and BERNFELD, l. c.) the  $\text{Na}^+$ -ions will cross the membrane faster than the  $\text{Cl}^-$ -ions and thus lower the positive charge on the inside. It is quite clear that this transfer of positive charges will be larger the larger the forces are which tend to move outwards the  $\text{Na}^+$ -ions. Therefore a large concentration difference (fig. 4 curve I) or a high P.D. (the inside strongly positive relative to the outside) will be followed by a considerable initial fall in P.D.

If on the other hand the  $\text{Na}^+$ -concentrations are equal on both sides and at the same time the P.D. is low (fig. 5, II to IV) the fall in P.D. as a consequence of the difference in the movements of cations and anions should be expected to be less which was exactly the case.

The secondary rise in the potential can hardly be ascribed to any diffusion potential. In a preliminary report (USSING, 1948) I suggested that the rise was due to an increase in  $\text{Cl}^-$ -permeability setting in some time after the increase in  $\text{Na}^+$ -permeability. This explanation might have been accepted but for the results with Ringer on both sides of the skin. Here a more free diffusion of  $\text{Cl}^-$  (as well as of  $\text{Na}^+$ ) would tend to lower the P.D.; and whereas curve I fig. 5 could be in accord with this view, all the other curves show a secondary P.D. rise which exceeds the initial fall. The secondary P.D. rise thus cannot originate from the diffusion of  $\text{Cl}^-$ . We shall return to this point later.

Whereas all the facts known point to an increase in  $\text{Na}^+$ -permeability following adrenaline application, the evidence as to an eventual increase in  $\text{Cl}^-$ -permeability is of a more indirect nature.

To be sure, after adrenaline application  $\text{NaCl}$  is seen to pour through the skin if the outside concentration is low; but at the same time there is a violent drop in the positive charge on the inside.

If, now, it is the P.D. which normally keeps the  $\text{Cl}^-$ -ions from running out, a P.D. drop will automatically lead to a leakage of  $\text{Cl}^-$ -ions. In the foregoing chapter we learned that the  $\text{Cl}^-$ -influx was not much changed after adrenaline addition. This may suggest that the  $\text{Cl}^-$ -permeability has increased since this would allow the influx to be unaltered when the attracting force, the R.D. ~~the R.D. force~~ down.

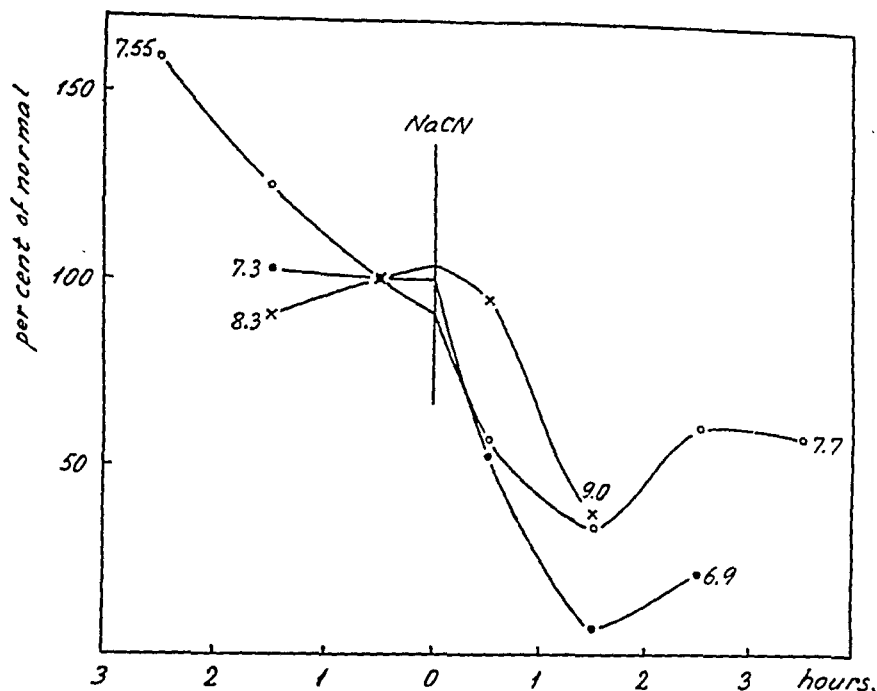


Fig. 6. Influence of cyanide on the  $\text{Na}^+$ -influx through the isolated frog skin. Ordinate: relative  $\text{Na}^+$ -influx (last period before  $\text{NaCN}$ -addition is put equal to 100 %). Abscisse: time in hours. At vertical line addition of  $\text{NaCN}$  to inside solution to make 4 mmol. At the beginning and end of each curve are given the pH values at the corresponding times.

### The Influence of Cyanide Poisoning on the Influx of $\text{Na}^+$ .

The experiments were performed in the following way: The  $\text{Na}^+$ -influx through the skin was determined in two or more normal periods. Then 0.5 ml 80 mmolar  $\text{NaCN}$ -solution (previously adjusted to a pH near that of the inside solution) was added on the inside and the  $\text{Na}^+$ -influx was followed until the fall in P.D. had ended which always follows cyanide poisoning. Examples are given in fig. 6.

It is seen that the  $\text{Na}^+$ -influx goes down and attains a minimum in the second one hour period after the poisoning. Later the influx climbs again, possibly due to a *post mortem* increase in cell permeability. These results are in good agreement with the view that the major part of the  $\text{Na}^+$ -influx is normally due to active uptake which can be poisoned with cyanide. It ought to be mentioned that cyanide does not block the outflux of  $\text{Na}^+$ . A leakage of salt can be observed after  $\text{NaCN}$ -poisoning.

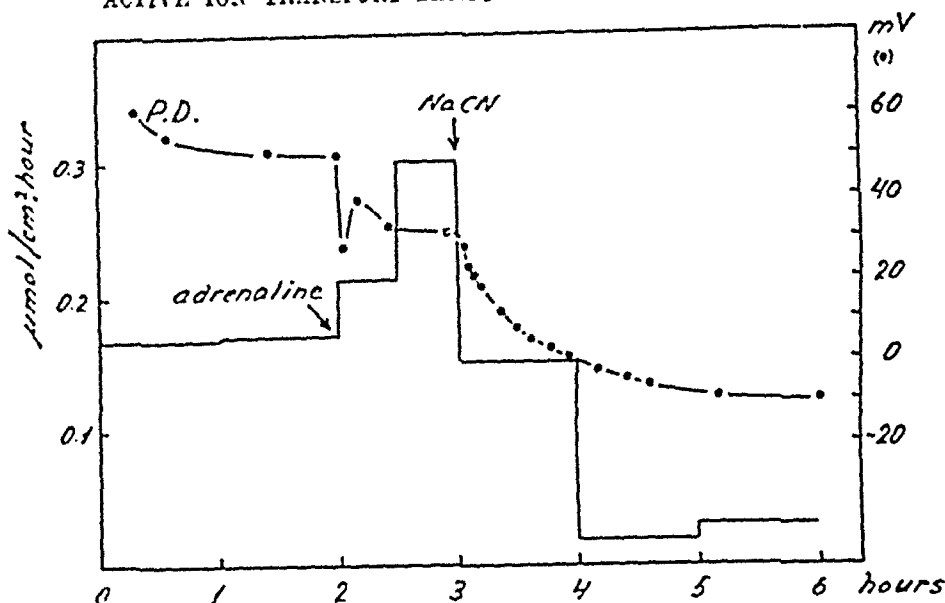


Fig. 7. Influence of the successive addition of adrenaline and cyanide on the  $\text{Na}^+$ -influx through and on the P.D. across the isolated frog skin. P.D. curve with dots,  $\text{Na}^+$ -influx: square line curve.

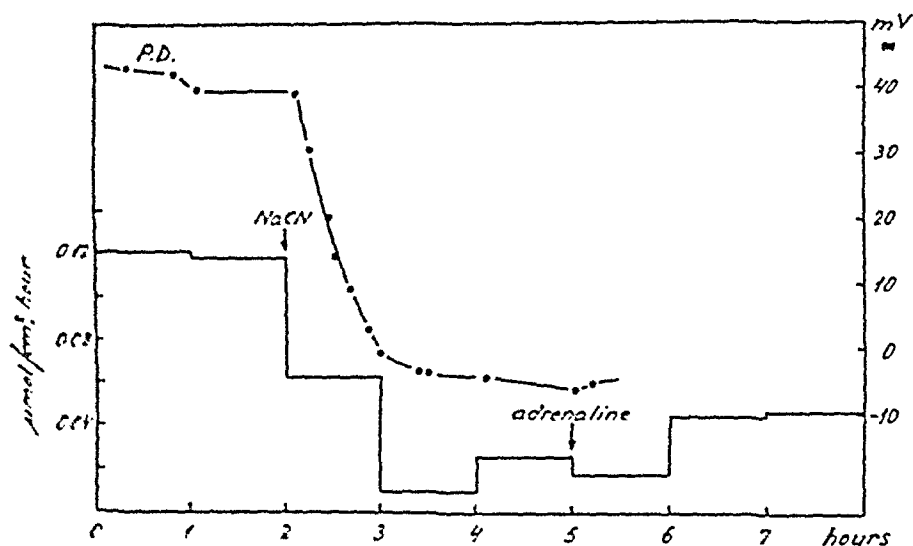


Fig. 8. Influence of the successive addition of cyanide and adrenaline on the  $\text{Na}^+$ -influx through and the P.D. across the isolated frog skin. P.D. curve with dots,  $\text{Na}^+$ -influx: square line curve.

Although cyanide as well as adrenaline induce a fall in P.D. and a leakage of salt, their actions are entirely different. This is most clearly illustrated in fig. 7 and fig. 8. The  $\text{Na}^+$ -influx and the P.D. are given as functions of time.

In the experiment shown in fig. 7 adrenaline is added at the first arrow. It is seen that the  $\text{Na}^+$ -influx increases and the P.D. shows the characteristic drop and secondary rise and a slow decline. At the second arrow NaCN is added and now both  $\text{Na}^+$ -influx and P.D. fall off, the  $\text{Na}^+$ -influx to reach a minimum in the second one hour period, the P.D. reaching a little later a constant value at  $-10$  mV.

Fig. 8 shows the reverse experiment. NaCN is given first and the P.D. as well as the  $\text{Na}^+$ -influx fall off. The addition of adrenaline now has no effect.

## Discussion.

1. Disregarding experiments with cyanide-poisoning or adrenaline stimulation we find that the influx of  $\text{Na}^+$  exceeds the outflux at all outside concentrations studied here viz. from 1—115 mmoles NaCl. When Ringer is applied on both sides of the skin the mean outflux is less than 10 % of the mean influx. The excess influx takes place against a P.D. of 30—110 mvolts. Thus it is justified to regard this influx to be due to active uptake. There still remains some 10 % of the influx to be accounted for. This influx may originate from active uptake too, it may represent classical diffusion or it may represent exchange diffusion (compare p. 2). At low outside concentrations the part played by free diffusion in the influx of  $\text{Na}^+$  is bound to be rather insignificant. On the other hand influx and outflux come nearer to each other, so that there is an increase in the proportion of the total influx which may or may not originate from exchange diffusion.

Is it so then, that we can regard the  $\text{Na}^+$ -influx as equal to the active uptake at high outside concentrations and equal to or smaller than the uptake at low outside concentration?

In order to answer this question it will be useful to approach the problem from a different angle:

If it were possible to stop the active uptake without changing the diffusion conditions it might be possible to study the changes in influx and outflux of ions so induced and to calculate from these data the true active uptake.

Although we know that cyanide does stop the active uptake of salt (HUF, l. c.) as well as it reduces the  $\text{Na}^+$ -influx appreciably (see p. 22), NaCN-poisoning might well have effects on cell permeability too. If, for a moment, we neglect this possibility we would

infer that cyanide poisoning would result in a salt loss at a rate which, at least in the beginning, would correspond to the true rate of active uptake minus the net uptake before the poisoning.

As an example we may take an actual experiment. The outside salt concentration was 5.48 mmol.; the Na-influx before poisoning  $0.1 \mu \text{ mole/cm}^2 \text{ h.}$ ; salt loss after poisoning  $0.44 \mu \text{ mole/cm}^2 \text{ h.}$ ; from this it would appear that the Na-influx far from giving too high results for the active uptake does actually give a result which is about 4 times too low. While this result might be due to an increase in cell permeability, it does not mean that such a permeability change is bound to have taken place as the following example will show. We may consider the following system (see fig. 9). The cell C is separated from the outside medium by the

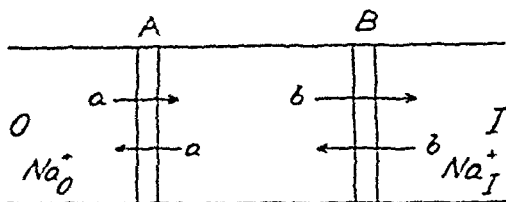


Fig. 9. Diagram of a system with two membranes presenting a high diffusion resistance (see text).

wall A and from the inside by the wall B. We assume ideal mixing in C as well as in O and I. We further assume the system to be in a steady state as to  $\text{Na}^+$  so that the  $\text{Na}^+$ -concentrations in each phase is kept constant. This is brought about by the active transfer of  $\text{Na}^+$  across B, so that the flux in both directions through B is  $b$ . Through A the flux in both directions due to diffusion is  $a$ . For the sake of simplicity the walls are taken to have unit area. If we want to know the flux of ions from I to O, this can be calculated as follows. Ions originating from I are designated  $\text{Na}_I^+$  and those from O  $\text{Na}_O^+$ . Because  $b$  moles of  $\text{Na}_I^+$  enter C per unit time the steady state demands that  $b$  moles of  $\text{Na}_I^+$  should be removed.

The  $\text{Na}^+$  will leave partly through A and partly through B in proportion to the flux from the cell in the two directions.

Thus we have that out of the  $b$  moles entering C from I in unit time

$$b \cdot \frac{a}{a + b} \text{ moles of } \text{Na}_I^+ \text{ leave through A}$$

$$\text{and } b \cdot \frac{b}{a + b} \text{ go back through B per unit time.}$$



The flux from O to I (and from I to O) is thus  $\frac{a \cdot b}{a + b}$ . Only in case  $a$  is very large compared with  $b$  will the flux from O to I (and vice versa) be a measure of the active transport through B. In all other cases the flux between O and I is smaller than the active transport.

As soon as, in an organ performing active transport, more than one membrane in the track of the ions represents a high diffusion resistance we must be prepared to encounter the phenomenon outlined above. The considerations are of course valid, *mutatis mutandis*, even if a net uptake or a net loss takes place.

This phenomenon may be the reason why we find the apparently high efficiency in the active transport through the frog skin, where under favourable conditions the net uptake is 90 % of the total  $\text{Na}^+$  influx so that only 10 % is left which could be exchange diffusion or ordinary diffusion. In contrast to this the exchange of  $\text{Na}^+$  between the isolated frog sartorius and the surrounding solution is of such magnitude that exchange diffusion is most likely to play an important part (USSING, 1947; LEVI and USSING, 1948). In the muscle we probably have to deal with only one really high resistance to the passage of ions, namely the cell membrane, whereas in the frog skin the number of layers offering a high resistance to ion movements cannot possibly be lower than two (see below).

We are thus lead to the following conclusion: At outside concentrations which are not too low the  $\text{Na}^+$ -influx — as determined by the tracer method — gives a fairly good measure of the rate of active  $\text{Na}^+$  transport across the skin. The method may, however, give much too low figures for the transport work performed by the skin because the internal diffusion losses are not included in the results. It remains a matter of definition whether eventual internal diffusion losses should be added to the net transport to give the total transport or whether such losses should be considered as an inherent part of the transport mechanism.

2. Whereas the sodium ions on either side of a living frog skin are not in thermodynamic equilibrium, so that it is necessary to assume an active transport, the situation is different for the  $\text{Cl}^-$ -ions.

If we take a case where the outside salt concentration is say 5 mmolar, the equilibrium condition is that the P.D. should be

$$58 \cdot \log \frac{115}{5} = 80 \text{ mV.}$$

This is a quite reasonable value for the P.D. especially if the inside pH is high, for example 8.3.<sup>1</sup>

The above consideration is only valid if the  $\text{Cl}^-$ -ions are able to cross the skin as free ions. This is entirely possible; we know of several cell membranes which are permeable to  $\text{Cl}^-$  though extremely little permeable to  $\text{Na}^+$  (Corpuscle membrane; muscle fibre membrane, see COXWAY 1947). It may speak in favour of the  $\text{Cl}^-$  being able to cross the skin in the free state that the variations in  $\text{Cl}^-$ -influx go parallel with the variations in P.D.

If a net uptake of  $\text{Cl}^-$  is to take place the potential difference should of course be higher than that calculated for the equilibrium. Unfortunately we have no determination of the outflux of  $\text{Cl}^-$ , so we only know for certain that (in the isolated skin) a net uptake takes place when both sides of the skin are bathed with Ringer (HURF, c.) and sometimes even if the outside solution is half Ringer (BARKER-JØRGENSEN, unpublished). We do know, however, that the  $\text{Cl}^-$ -influx is much lower than the  $\text{Na}^+$ -influx in most cases, and whereas the  $\text{Cl}^-$ -influx is the absolute maximum for the net  $\text{Cl}^-$ -uptake we know that the  $\text{Na}^+$ -influx is as a rule not very far from the net  $\text{Na}^+$ -uptake.

Two possibilities present themselves to explain the difference between the  $\text{Na}^+$  and the  $\text{Cl}^-$  uptake:

1) The outside solution receives other cations instead of the  $\text{Na}^+$ -ions removed in excess over the  $\text{Cl}^-$ .

2) One or more acids are formed in the cells, the anions of which follow the  $\text{Na}^+$  into the inside solution, whereas the  $\text{H}^+$ -ions (or other cations) replace the outside  $\text{Na}^+$  removed in excess.

As to point 1) the outside solution might receive  $\text{H}^+$ ,  $\text{K}^+$  or  $\text{Ca}^{++}$ ; these are probably the only cations available in sufficient amounts. (As a more remote possibility we might think of  $\text{NH}_4^+$  formation in the skin.)

A calculation shows that in cases where the  $\text{Na}^+$ -influx was most in excess over the  $\text{Cl}^-$ -influx the outside solution would receive per hour and per ml about  $2 \times 10^{-6}$  meqv. of other cations to replace the sodium transported in. It is easily seen that even a moderate amount of buffer substance in the outside solution might veil a  $\text{H}^+$ -excretion of that magnitude, at least in short experiments. There is certainly a tendency towards acidification

<sup>1</sup> When living frogs take up salt, say from a 0.01 mmolar solution a P.D. higher than 240 mV would be demanded. Such values have not been observed on the isolated skin, but the possibility remains that there is also a specific transport of  $\text{Cl}^-$ , which may be more active in the living animal.

of the outside solution in several cases. Systematic experiments are planned, however, to find out the rôle of  $H^+$ -ions in replacing the  $Na^+$ -ions taken up.

According to KROGH (1938) frogs do not take up  $K^+$  and  $Ca^{++}$  through the skin. On the other hand he found that at least  $Ca^{++}$  is continuously lost through the skin. All losses of cations during the active uptake of  $Na^+$  will act as a short circuit of the P.D. and may thus diminish the uptake of  $Cl^-$ .

Possibility 2) depends on the formation of acid within the cells. The anions follow the  $Na^+$  into the inside solution, whereas the  $H^+$ -ions diffuse out to take the place of  $Na^+$ .

In its extreme case when the  $Cl^-$ -permeability is nil such a mechanism would turn the outside solution into hydrochloric acid.

Parenthetically it might be worth saying that the acid formed need not be an organic acid. In the first place  $CO_2$  is formed by the epithelial cells themselves. If carbonic anhydrase is present in the cell  $CO_2$  may moreover diffuse into the cell from the inside solution, being turned into  $HCO_3^-$  and leave again as such.

3. *Remarks on the nature of the active  $Na^+$ -transport.* Active transport may be spoken of when a substance passes from one phase into another at a rate which is higher than can be accounted for by diffusion (or exchange diffusion). However, it may here be convenient to restrict the use of the term active transport to cases where the substance in question attains a higher potential energy when passing from one phase into another. This of course takes place at the expense of potential energy derived from processes going on in the membrane or the organ separating the phases.

Taking the  $Na^+$ -transport in the frog skin as an example it is not very likely that the  $Na^+$ -ions should pass in all the way as free ions against a potential — as well as a concentration gradient. Now, of course, it would be audacious to base conclusions concerning the state of  $Na^+$  while passing a membrane solely on observations on a rather complex structure like the frog skin.

If the passage of  $Na^+$  as free ions is unlikely in the frog skin it is, however, still more unlikely that  $Na^+$  should cross the cell membrane of for instance muscle fibre or nerve in the free state.

In both these cell types (as in most animal cells) the  $Na^+$ -concentration is low. Yet we know that  $Na^+$  does penetrate (KROGH 1946) and an active extrusion must take place. In the resting state the content of these cells is known to have a considerable negative charge relative to the outside solution, so that we have

a situation which very much resembles the frog skin case.  $\text{Na}^+$  is carried out against both the potential and the concentration gradients. But here the  $\text{Na}^+$ -ions in the cell and in the surrounding solution are only separated by one thin cell membrane. The most reasonable explanation of the passing out of  $\text{Na}^+$ -ions under these conditions is that they do so as uncharged complex or as ion pairs.

This is the more likely because we have found (USSING, 1947, LEVI and USSING, 1948) that considerable exchange diffusion of  $\text{Na}^+$  is practically bound to go on in the isolated muscle, and as discussed in the introduction (p. 4) exchange diffusion depends upon some form of binding of the ions taking part in the exchange.

An exchange diffusion system may participate in an active transport in different ways:

One possibility is exemplified in the following hypothetical system. Let us consider the skin of a fresh water animal consisting of two layers of cells. The membrane separating the cell layers is regarded to be essentially lipoid nature but containing a number of anions  $\text{B}^-$  (see the introduction p. 3) allowing exchange of certain cations. We now assume that the outer cells synthesize acetylcholine. According to BARNES and BEUTNER (1947) the acetylcholine ions are relatively soluble in lipoids and will therefore diffuse into the inner cells. Here the acetylcholine is split by choline esterase into choline and acetic acid. The choline ions cannot diffuse back through the lipoid layer due to the hydroxy group, whereas the acetic acid can diffuse back. The inner cells therefore attain a positive charge which will lead to a diffusion in of some anions, for instance  $\text{Cl}^-$ .

The choline ions which could not go back by simple diffusion are supposed to form a complex with the  $\text{B}^-$ -anions, thus enabling them to go back by exchange diffusion. If the choline concentration is kept extremely low in the outer cells by acetylcholine synthesis, choline ions can only go back by exchange diffusion if they are replaced by other cations which are also able to participate in the exchange diffusion, for example  $\text{Na}^+$ . Thus the net result would be the transfer of one molecule  $\text{NaCl}$  from the outside to the inside at the expense of the splitting of one molecule acetylcholine.

It should be emphasized that this is only a model system. However, it might be worth while to consider its possible realisation in organs where its components are present, for instance in nerve, muscle and electrical organs. Other systems where ex-

change diffusion may participate in active transport can be realized if the anion of the complex  $BNa$  on arrival to one of the sides is transformed into a substance which cannot form complexes (for example by oxydation or reduction), whereas  $B^-$  is reformed on the other side.

For the following considerations it is a matter of indifference how the  $Na^+$  is set free from the unknown complex. The only assumption is that a necessary step in the active transport of  $Na^+$  is the formation of a complex  $BNa$  in which form  $Na^+$  is supposed to cross the membrane.

4. *The site of the active  $Na^+$ -transport in the frog skin.* If we wish to localize the site of active transport in the frog skin it may be useful to recapitulate shortly the histology of the organ. The skin consists of two layers of different origin, namely the mesodermal chorion and the ectodermal epithelial layer (see fig. 10). The chorion is built up of connective tissue containing blood vessels and smooth muscle cells. Just beneath the epithelial layer the chorion contains a number of chromatophor cells. The whole structure is a meshwork presenting no serious obstacles to the diffusion. The active transport cannot take place here.

The epithelial layer consists of only two or three layers of cells. Nearest to the chorion we find the stratum germinativum of cylindrical cells; then come cells of which some may resemble normal epithelial cells whereas others are being keratinized, and finally nearest to the outside the layer of fully keratinized cells.

From a histological point of view the active transport must evidently be ascribed to the stratum germinativum, possibly assisted by the still normal cells lying further out. In many places, however, the stratum germinativum cells are the only living ones between the surface and the chorion.

It may perhaps be possible to locate the active transport still better: We know that pH changes in the inside solution result in conspicuous changes in P.D. (MEYER and BERNFELD l. c.) and in influx of  $Na^+$  (see p. 12). On the outside, on the other hand, pH may be varied within rather wide limits before influencing P.D. and  $Na^+$ -influx. Thus the site of transport can be reached by diffusion more easily from within than from without.

Similarly adrenaline exerts no influence at all on salt permeability (BARKER JØRGENSEN l. c.),  $Na^+$ -influx or potential, when added on the outside, whereas it has a drastic effect on all three when added on the inside.



Fig. 10. Cross section of the skin of the frog (*Rana temporaria*).

As the chorion can be left out of discussion this means that pH change and adrenaline exert their influence on the stratum germinativum and probably on that surface of the cells which is in contact with the chorion. If this view is correct, it means that the cells of the stratum germinativum extrude  $\text{Na}^+$ -ions (and  $\text{Cl}^-$ -ions) through the basal cell membrane. As we have seen already, most animal cells must extrude  $\text{Na}^+$ . The difference between the epithelial cells and other cells thus is not that they extrude  $\text{Na}^+$ , but that they do this only on the side turning against the animal and not on the side turning outwards.

5. *The factors determining the rate of active  $\text{Na}^+$ -transport.* What is the origin of the potential measured across the frog skin? From what we have seen here it seems very likely that the positive charge of the inside relative to the outside is due to the active  $\text{Na}^+$ -transport. As  $\text{Na}^+$ -ions are transferred across the skin more

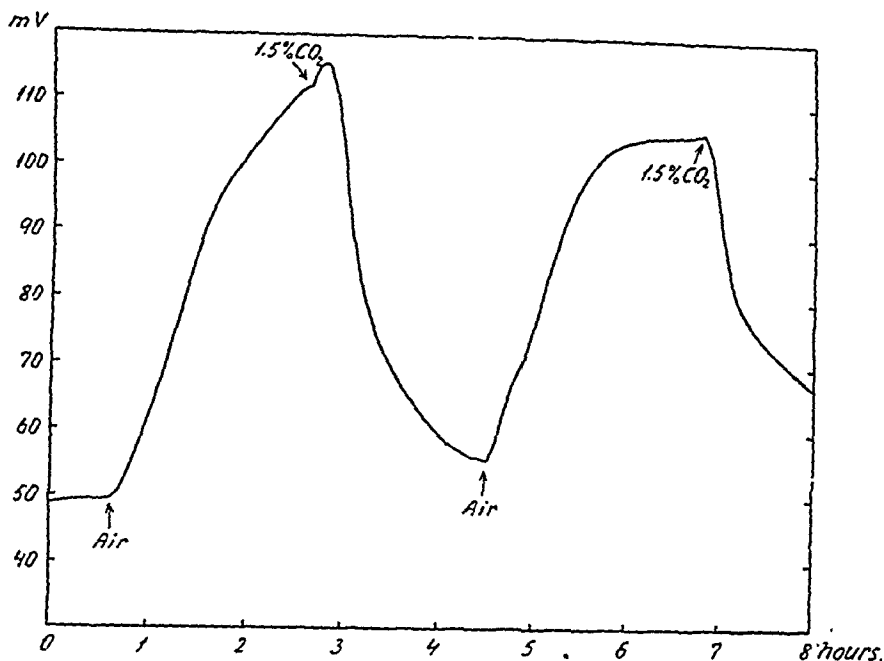


Fig. 11, Influence of CO<sub>2</sub>-tension on the P.D. across the isolated frog skin with Ringer on both sides. The arrows indicate the times for change from air mixing to mixing with 1½ % CO<sub>2</sub> or vice versa. pH of the solutions (both inside and outside) with air mixing 8.1 and by 1½ % CO<sub>2</sub> mixing 6.9.

rapidly than the Cl<sup>-</sup>-ions can follow the inside will be charged positively. This view is supported by Hur's (l. c.) important finding that monobromacetic acid which lowers the potential across a frog skin, soaked on both sides with Ringer, will also depress the active salt transport. The addition of lactate may partly restore the potential during monobromoacetate poisoning and this substance, too, keeps the active transport going.

We have now learned that also factors like outside salt concentration and inside pH affect potential and Na<sup>+</sup>-influx in a uniform way. As the potential can not possibly be the reason for the Na<sup>+</sup>-influx it may be permissible to assume that the potential is due to the Na<sup>+</sup>-transport. If this hypothesis is tentatively accepted, how can it be then that a shift of the inside pH, say, one unit to the alkaline side makes the inside c. 50 mvolts more positive? According to the hypothesis the increase in potential should be due to an increased Na<sup>+</sup>-transport, but this should not necessarily result in an increase in potential indicating the change in H<sup>+</sup>-activity as if the skin were a glass electrode.

The most reasonable explanation is that the skin (or rather the cell membrane of the stratum germinativum cells) is in fact acting

as a glass electrode (compare MEYER and BERNFELD l. c.) being much more permeable to  $H^+$ -ions than to other ions. The necessary condition for our measuring a shift of 60 mV for the shifting of pH one unit is evidently that the pH of the cytoplasm of the transporting cells is being kept constant.

Thus we arrive at the conclusion that the  $Na^+$ -transport proceeds at a rate exactly necessary to keep the pH of the cells constant.

The question may be asked whether active  $Na^+$ -transport is at all suited to bring about a lowering of pH in the cells. The answer is that active transport of  $Na^+$  means in fact a forced exchange of  $Na^+$  against  $H^+$ . The regulation of the transport so as to achieve this goal might of course be vaguely described as biological regulation. This is, however, unnecessary. If the first step in the active  $Na^+$ -extrusion is the formation of a complex  $BNa$ , and if the amount of complex present is the pace setting factor for the transport, we will have a quite automatic regulation of the transport because the amount of complex present at any time is strongly dependent on pH (see p. 3). Irrespective of the chemical nature of the anion  $B^-$  we have

$$\frac{[BNa]}{[BH]} = \frac{[Na^+]}{[H^+]} \cdot \frac{K_H}{K_{Na}}$$

If the  $Na^+$ -concentration is considered as constant we have

$$\frac{[BNa]}{[BH]} = \frac{K}{[H^+]}$$

where  $K$  is a constant. Only in case the affinity between  $B^-$  and  $Na^+$  is much higher than the affinity between  $B^-$  and  $H^+$  will this dependence of pH be without significant influence on the total amount of  $BNa$ . But this case is extremely unlikely because  $H^+$  is generally known to be the best complex former of all cations.

The pH which is of importance for the extrusion of  $Na^+$  from cells is evidently that prevailing inside the cell membrane. This is in good agreement with the fact that, in contrast to the rapidly diffusing  $CO_2$ , the slowly diffusing phosphate buffers take a fairly long time to provoke a shift in  $Na$ -influx and potential.

The  $Na$ -influx and potential also show a parallel dependence on outside  $NaCl$  concentration. This can be easily understood if we assume that the  $Na^+$  extruded from the cells is replenished by diffusion from the outside. Then it is plain that the rate at which



$\text{Na}^+$  is allowed to enter the transporting cells will be a very important factor in determining the rate of active transport.

6. *The nature of the adrenaline reaction.* How does our knowledge concerning the adrenaline reaction fit into the picture we are forming of the structure of the frog skin? If the outside  $\text{NaCl}$ -concentration is lower than that on the inside, we observe on adding adrenaline to the inside solution 1) a large net salt leakage, 2) a fall in P.D. followed immediately by a smaller rise, 3) an increase in  $\text{Na}^+$ -influx to about double its former value. From what has been said already it is obvious that adrenaline must increase the permeability of the basal cell membrane of the transporting cells. The increase is evident towards  $\text{NaCl}$ , phosphate (determined with radio-phosphorus, unpublished) and glucose (GELLHORN and GELLHORN, 1929). A permeability change is, however, not sufficient to account for the reaction.

In experiments with Ringer on both sides of the skin, adrenaline leads to a rise of the potential above the resting value. This indicates that the increased influx through the basal part of the cell is in part at least due to increased active transport. But this is just what could be expected when an increase in the permeability of the cells makes possible a penetration of the buffer systems from the inside solution into the cells thus moving their pH in alkaline direction (although we do not know the pH of the cells it is hardly as alkaline as 8.3 at which pH most of our experiments were performed).

### Concluding Remarks.

It may be permissible to give a comprehensive representation of the view we have arrived at concerning the active ion transport through the frog skin, even if some of its viewpoints require further experimental evidence. The reason and excuse for doing so is that a clearly formulated hypothesis may be useful in the planning of future experiments.

The transport is assumed to be performed by the cells in the stratum germinativum. The cells have a membrane which normally is very little permeable to ions (except  $\text{H}^+$  and possibly  $\text{OH}^-$ ). Whereas the mechanism for active extrusion of  $\text{Na}^+$  which is present in most cells is degenerated or inoperative in the cell membrane on the distal end of these cells it is still active at the basal part. This leads to asymmetrical extrusion of  $\text{Na}^+$  towards

the inside, resulting in a positive charge here relative to the outside. This positive charge attracts  $\text{Cl}^-$ -ions which move in, as a rule in somewhat smaller amount than the  $\text{Na}^+$  so that some cations ( $\text{H}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ ) must go out and some anions formed in the cells must follow the  $\text{Na}^+$ -ions in. The  $\text{Na}^+$ -ions pass through the basal cell membrane of the transporting cells as an uncharged complex  $\text{BNa}$  from which the  $\text{Na}^+$  is set free on arriving at the outside of the membrane by a process requiring chemical energy derived from the oxydative metabolism.

The anion  $\text{B}^-$  which forms the complex does not form a similar complex with  $\text{K}^+$  or at least is the affinity much smaller between  $\text{K}^+$  and  $\text{B}^-$  than between  $\text{Na}^+$  and  $\text{B}^-$  as shown by the fact that frogs do not take up  $\text{K}^+$ . On the other hand  $\text{B}^-$  combines with  $\text{H}^+$  to form  $\text{HB}$ . This fact will tend to keep the pH of the cells constant because a decrease in  $\text{H}^+$ -concentrations within the cells will lead to the formation of more  $\text{BNa}$  with a consequent increase in  $\text{Na}$ -extrusion, a process which will lower the pH. Changes in the cellular pH are therefore reflected in the rate of active  $\text{Na}^+$ -transport and in the P.D. across the membrane. The  $\text{Na}^+$  and  $\text{Cl}^-$  carried to the inside solution is replenished by diffusion from the outside. The ionic permeability of the outside layer of the skin therefore exerts an important influence on the rate of transport and also on the P.D.

Adrenaline increases enormously the permeability of the transporting cells, but at the same time stimulates the active transport perhaps by displacing the cellular pH in alkaline direction. The adrenaline reaction thus resembles very much the "pricking reaction", the curious increase in outflux as well as influx of sodium ions which was observed in the axolotl as the result of injury to the skin of the animals (BARKER JØRGENSEN, LEVI and USSING, 1946).

### Summary.

1) Experiments with radio-sodium ( $\text{Na}^{22}$ ) show that when an isolated frog skin separates two solutions containing  $\text{NaCl}$  (that on the inside being Ringer) the  $\text{Na}^+$ -flux from outside to inside is normally much higher than the outflux; this is true even when the outside  $\text{NaCl}$  concentration is only one millimole/l.

2) The  $\text{Na}^+$ -influx (and thus the active sodium transport) shows

a pronounced dependency on the pH of the inside solution. High pH values give high values for the  $\text{Na}^+$ -influx and vice versa.

3) The pH of the outside solution has little effect on the  $\text{Na}^+$ -influx until pH is below ca. 5. Then the  $\text{Na}^+$ -influx drops to a very low value, whereas the  $\text{Cl}^-$ -influx goes up.

4) The  $\text{Cl}^-$ -influx is as a rule lower and often much lower than the simultaneous  $\text{Na}^+$ -influx. The variations in the  $\text{Cl}^-$ -influx generally go parallel to the variations in  $\text{Na}^+$ -influx. There is also a pronounced parallelism between the potential difference across the skin and the influx of  $\text{Na}^+$  and  $\text{Cl}^-$  so that the more positive becomes the inside relative to the outside, the higher is the influx of  $\text{Na}^+$  and  $\text{Cl}^-$ .

5) When added on the inside of the skin adrenaline in the concentration  $1/10^6$  brings about an enormous increase in the outflux of  $\text{Na}^+$  and a considerable increase in the influx of this ion. Immediately after the addition of adrenaline a violent drop in the P.D. is observed. This drop and the further course of the P.D. curve depends on the outside salt concentration and the magnitude of the P.D. before the adrenaline addition. It is concluded that the initial drop in P.D. is due to an increased permeability to  $\text{Na}^+$ . The secondary rise in P.D. shortly after the drop must be due to an increased  $\text{Na}^+$ -transport.

6) Cyanide poisoning reduces the  $\text{Na}^+$ -influx to 5—25 % of the original value. The outflux of ions is not reduced.

7) The theoretical basis for the use of tracers to determine the active transport of an ion species through a membrane is considered. In the frog skin the  $\text{Na}^+$ -influx is as a rule a fairly good measure of the  $\text{Na}^+$  actually transferred across the skin. But the total transport work performed by the transporting cells is greater than indicated by the flux because, due to back diffusion, some  $\text{Na}^+$  ions may be subjected to transport more than once before leaving the skin.

8) Based on the experiments a hypothesis is put forward which will account for the interdependency between the in- and outflux of  $\text{Na}^+$  and  $\text{Cl}^-$  and the potential difference across the skin.

The radioactive isotopes used were kindly provided from the Institute of Theoretical Physics in Copenhagen. I wish to extend my gratitude to the head of this institute, Professor N. Bohr and to his staff, notably Dr. O. Lassen who prepared the radioactive samples.

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From the Laboratory of Zoophysiology, University of Copenhagen.

## **The Influence of the Corticotropic Hormone from Ox on the Active Salt Uptake in the Axolotl.**

By

VALBORG KOEFOED JOHNSEN and HANS H. USSING.

Received 21 June 1948.

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### **Introduction.**

In a previous study (BARKER JØRGENSEN, LEVI and USSING, 1946) it was found that a preparation of the pressor principle from the hind lobe of the ox hypophysis (Insipidin) when injected into axolotls induced an increased uptake of salt through the skin of the animals. It seemed strange, however, that the antidiuretic hormone, in higher vertebrates inducing a fall in osmotic pressure of the blood in the axolotl, calls forth a salt uptake leading to an increase in osmotic pressure. Moreover, it is known (WHITE, 1944) that hind lobe preparations may show a corticotropic effect. It was therefore considered necessary to test whether the corticotropic hormone proper has an effect on the salt uptake comparable with that of Insipidin. Beforehand, it seems quite likely that the hormones from the adrenal cortex, exerting so pronounced an effect on the salt reabsorption in the kidney tubules, also increase the salt uptake through the skin and it will be seen from the following that the experimental evidence is in favour of this assumption.

### **Preparation of the Hormone.**

20 g of desiccated beef pituitary powder were extracted for two hours with 125 ml 55 % acetone acidified with 5 ml of 5 mol HCl. This extraction was repeated 3 times. Crude prolactin was then

prepared according to the method described by LYONS (1937) with the modifications suggested by WHITE, BONSNES and LONG (1942).

Further fractionation was made according to the method described by SAYERS, WHITE and LONG (1943).

On lowering the pH of the dialyzed *fraction N* to pH 4.7 no precipitate appeared unless acetone was added to a concentration of 50 %. The precipitate was washed with acetone and dried in vacuo over concentrated sulfuric acid.

In three cases a *fraction N* which had been stored for 6 months with  $\text{NH}_4\text{OH}$  was used and this proved as effective as the ordinary preparation.

Each experimental animal received about 1—2 mg of the hormone preparation dissolved in  $\frac{1}{2}$  ml of Ringer solution, while the controls had  $\frac{1}{2}$  ml of pure Ringer solution.

The technique is the same as described by BARKER JØRGENSEN, LEVI and USSING (1946) with the single exception that also during the experiments the animals were kept in tap water.

## Results.

### 1. The Chloride Balance after Injection of the Corticotropic Hormone.

Immediately after an injection, whether of corticotropic hormone dissolved in Ringer or of Ringer, the animals as a rule show a salt loss lasting for several hours (see fig. 1). A similar salt loss results from any damage to the skin of the animals (pricking effect, JØRGENSEN, LEVI and USSING, l. c.). After the fading of this pricking effect the animals receiving the hormone gain chloride and this process continues for about three days; thereafter the salt loss exceeds the uptake. In the control animals receiving Ringer we generally observe a slow but steady loss of salt. The duration of the reaction to the corticotropic hormone is about the same as that to the pressor principle.

The reactions shown in fig. 1 may be regarded as typical, though control animals sometimes show a spontaneous uptake, and the hormone reaction is of varying strength. The diagram shown in fig. 2 will facilitate the comparison between the hormone-injected animals (16) and the controls (10). The abscissa represents the gain of chloride (in  $\mu\text{moles}$ ) between the 6th hour (when the pricking effect has disappeared) and the 72th hour after the injection, and the ordinate represents the number of animals showing an uptake of a given magnitude. A chloride loss is regarded as a negative uptake. All uptakes between two subsequent multipla of 100  $\mu\text{moles}$  are considered to belong to one class. It is evident

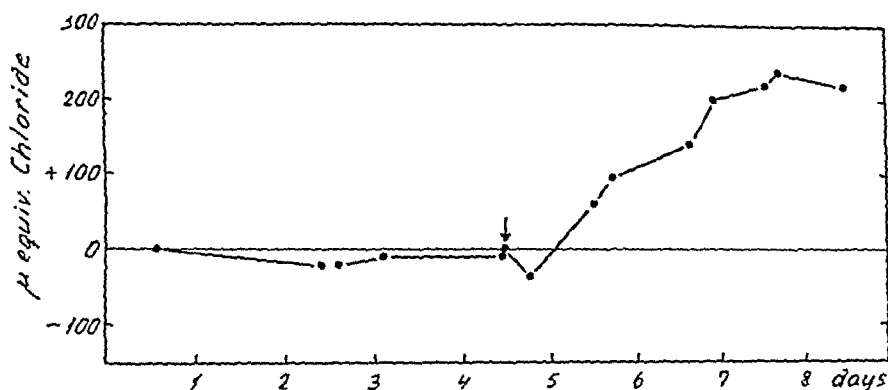


Fig. 1. Net salt uptake in an axolotl injected (at the arrow) with adrenocorticotrophic hormone.

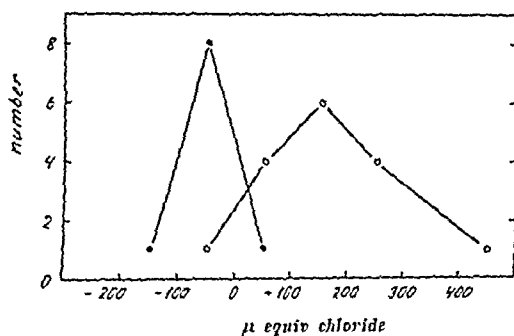


Fig. 2. Distribution curves showing the magnitude of chloride uptake in the animals.

●—● controls, ○—○ hormone-injected animals.

from this figure that the hormone has an effect and a mathematical treatment, a *t*-test, shows that the difference between the hormone-injected and the control animals is highly significant. Even if we choose a shorter period for a comparison of the two groups of animals the effect of the hormone must be regarded as fairly well established. Between the 24th and the 48th hour the hormone animals show a mean chloride gain of 55.8  $\mu$ moles whereas the controls show a mean loss of 22.5  $\mu$ moles. *t* for the difference is 3.66 which means that the probability for the difference between the two curves being not significant is only a few per mille.

## 2. The True Na-uptake as Measured with $\text{Na}^{24}$ .

The experiments mentioned have given good evidence that the corticotrophic hormone induces a net salt gain in the animals.

It has still to be found out whether this gain is due to an increased salt uptake, to a decreased salt loss, or to a combination of both effects. In a number of cases we have therefore determined the true active Na-uptake with  $\text{Na}^{24}$ . Although certain objections can be made against the general use of isotopes to measure the active transport of ions, a study of the Na-transport across the isolated frog skin has shown the isotope method to give a fairly good measure of the amount of Na which is *actually transported* across the skin (USSING 1948). The skin of the axolotl having a structure very similar to that of the frog skin it seems justified to use the method here too.

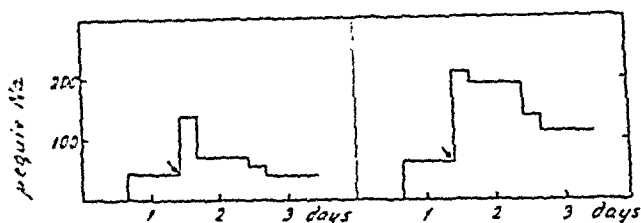


Fig. 3. The active uptake of sodium in axolotls.

Left: average of two control animals. Right: average of six hormone-injected animals.

In all cases the hormone injection results in an increase in Na-uptake of several hundred percent (see fig. 3) lasting for 1–5 days, whereas the control animals injected with Ringer only show a short lasting pricking effect or no increase at all.

It is thus seen that the corticotropic hormone calls forth an increase in the active Na-uptake. Whether the Na-reabsorption in the kidneys is increased or not it is impossible to tell from the material presented here *because the urine has not been collected*. The true Na-loss which could be found as the difference between the "true" Na-uptake and the net Na-uptake is thus the sum of the urinary Na, the Na which has diffused out, and the amount which may have been excreted through the skin glands.

### Discussion.

The experiments described show that it is possible from the anterior lobe of the pituitary to prepare a substance which on injection increases the active salt uptake in the axolotl. The pro-



cedure employed for the preparation makes it reasonable to assume that this substance is identical with the corticotropic hormone. Although this conclusion is very tempting no direct proof has been obtained that the reactions go via a stimulation of the adrenal cortex.

In a few experiments with desoxycorticosterone acetate the salt uptake as compared with that of the controls was not significant. This may partly be due to the fact that the mere manipulation with the animals causes a "pricking effect"; maybe, since the desoxycorticosterone acetate was placed as crystals in small pouches cut into the folds of the skin the treatment was rather hard. On the other hand, a net salt uptake was seen after the injection of thyroxine. Although only three animals were treated and the uptake was smaller than the mean uptake after the corticotropic hormone, this result is still worth mentioning because KOCH and HEUTS (1942) found that thyroxine feeding evokes a profound disturbance in the salt metabolism of the stickleback (*Gasterosteus aculeatus*). These authors assume the thyroxine reaction to be an indirect one via the adrenal cortex.

It is well known that a large variety of agents, for instance sex hormones and related substances, have a pronounced influence on the function of the cortex. In one explorative experiment we have in fact seen the injection of distilbestrole into an axolotl to call forth a considerable salt uptake. This result may be accidental, but distilbestrole belongs to the series of substances which are known to increase the production of hormone in the cortex and which also increase the salt uptake through the skin of the axolotl.

### Summary.

The influence of the adrenocorticotropic hormone on the salt balance in axolotls has been studied.

Injection of the hormone was found to increase the salt uptake through the skin of the animals.

The authors are greatly indebted to the Rockefeller Foundation for grants which have made this investigation possible.

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## Studies on the Biochemistry of Human Semen.

### III. The Viscosimetric Determination of Hyaluronidase.

By

FRANK LUNDQUIST.

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Hyaluronidase determinations have been performed in about as many ways as there are investigators in this field. The main principles employed in these test methods have been measurement of the spreading factor activity, the chemical determination of the acetyl glucosamine, glucuronic acid or reducing substances liberated, the prevention of turbidity or clotting of mucin solutions on acidification and the reduction of substrate viscosity (for references cf. MEYER 1947).

Of these principles that of viscosity measurement appears to be best suited for exact evaluation, the other methods being liable to various errors (MEYER *l. c.*, DORFMAN and OTT 1948), MADINAVEITIA and QUIBELL (1940) used the time necessary for reduction of the relative specific viscosity to half the initial value as a measure of the enzyme activity. This method has later been varied with respect to origin and concentration of substrate, pH and concentration of buffer and salts, each of these factors playing an important rôle for the rate of the enzymatic process. The principle of using the "half viscosity time" has in the present writer's opinion certain drawbacks:

1) The time of mixing the substrate and enzyme solutions must be well defined. This is not quite easy to obtain as the viscous substrate solution has little tendency to mix with the enzyme solution. CHAIN and DUTHIE (1940) rotate their samples for several minutes before the measurements are started, whereas

SWYER and EMMENS (1947) use squirting the enzyme solution into the substrate from a syringe through a fine (no. 26) needle, and thus apparently obtain reproducible results.

2) In order to obtain reliable viscosity measurements, viscosimeters with rather long flowtimes (for water) must be used. This introduces a serious error, especially in the beginning of the measurement, because the viscosity decreases during the individual measurements of the flowtime and it therefore becomes impossible to state exactly to which moment the measured viscosity corresponds. The generally adopted procedure is to use the starting time plus half the flowtime. This is evidently not correct though the error introduced may be small when the rate of viscosity reduction is not too high.

3) The magnitude of the initial viscosity which is essential for the determination of the half viscosity level is estimated through extrapolation from the curve. If the half viscosity point is to be reached within a reasonable time, the curve is very steep in the beginning and this together with the errors mentioned in 1) and 2) will often make the estimate of the initial viscosity rather unsatisfactory. This difficulty has been circumvented by DALGAARD-MIKKELSEN and KVORNING who make a separate measurement of the initial viscosity using inactivated enzyme solution. This procedure complicates the method and requires an extra sample of the enzyme solution to be tested. In the case of hyaluronidase determination on human semen, where material is often scarce and where a considerable viscosity is generally found making the correction necessary, this may be a serious drawback.

Apart from these purely practical objections, some considerations of a more general nature may be advanced. The use of the time necessary to halve the viscosity under specified conditions implies that the process is a first order reaction and that the viscosity is proportional to the substrate concentration. This question has been investigated by DORFMAN (1948), who decides that due to the small substrate concentration the first order scheme is only roughly approximated. He derives an expression which takes into account the diminishing substrate concentration and gives a velocity constant far better than the usual procedure. However, the inhibitory influence of the reaction products on the enzyme has not been accounted for. The conditions under which the experiments of DORFMAN were made were, moreover, rather unfavourable for the combination of enzyme and substrate, in

so far as the substrate concentration was lower than generally used in the viscosimetric assay of hyaluronidase. Also SWYER and EMMENS (1947) use small substrate concentration with the justification that under these conditions the relative specific viscosity and the hyaluronate concentration are roughly proportional. Their modification of the method using a fixed incubation time appears to be a definite improvement compared with the other methods.

### Preparation and Properties of Hyaluronate.

A comparison has been made between samples of potassium hyaluronate prepared from the sources most commonly applied, viz. vitreous humor from cattle eyes, synovial fluid from the astragalo-tibial joint of cattle and human umbilical cords. The eyes and synovial fluid samples were used as fresh as possible from the slaughter house, the umbilical cords were collected under acetone.<sup>1</sup> The procedures used for the preparation of hyaluronic acid from these sources were identical so far as possible, the main features being enzymatic removal of protein as recommended by ROBERTSON, ROPES and BAUER (1940) (in the case of synovial hyaluronate) and precipitation of the potassium hyaluronate with alcoholic potassium acetate acc. to MCCLEAN (1943).

#### a) *Synovial Fluid Hyaluronate.*

Portions of one liter of synovial fluid were filtered through a double layer of gauze and diluted with about 2 vols. of water. 1 per cent of the volume of glacial acetic acid was added under vigorous stirring with a glass rod. The mucin is precipitated as a stringy clot adhering to the rod. After washing with a little water the mucin precipitate is dissolved in about 200 ml of water under gradual addition of potassium hydroxide. Care is taken to keep the pH below 9. It is advantageous to cut the mucin in small pieces as the dissolution is a rather slow process. The pH is adjusted to about 8 and 0.5 per cent sodium bicarbonate is added together with 3 grams of pancreatin (B. D. H.) and a little toluene to prevent bacterial growth. Incubation at 37° for 24 hours now destroys the protein and leaves the hyaluronic acids intact. After thorough cooling 10 per cent trichloroacetic acid is added and the mixture filtered after standing for about 1 hour. The resulting solution is generally clear or nearly so. It is neutralized with potassium hydroxide and 1.3 volumes of a saturated solution of potassium hydroxide in alcohol are added under vigorous stirring. Potassium

<sup>1</sup> The author is indebted to the gynecological departments A and B of the Rigshospital, Copenhagen, for kindly placing this material at his disposal.

hyaluronate is precipitated as white stringy masses. When the solution is allowed to stand for half an hour in the refrigerator a flocculent precipitate is formed, which was also found to be potassium hyaluronate. After filtration and washing with alcohol the substance is dissolved in 200 ml of water and the precipitation with alcoholic potassium acetate is repeated. This time most of the substance is precipitated in the stringy form. It is washed first with alcohol containing a trace of acetic acid, then with alcohol, and at last with ether. The white or slightly grey substance is dried in vacuum over phosphorus pentoxide. Yield: about 300 mg. (Preparation no. 1, table 1.)

An experiment was made to prepare hyaluronate without incubation with proteolytic enzyme. The procedure was identical with that outlined above except that the mucin solution was treated directly with 10 per cent trichloroacetic acid. A copious, somewhat stringy, precipitate was formed which gradually (in the course of about an hour) became flocculent leaving a viscous solution. After centrifugation the supernatant was worked up as above. Yield: 200 mg. A quite considerable amount of hyaluronic acid is probably still present in the precipitate. No attempt was, however, made to increase the yield. (Preparation 2, table 1.)

#### b) *Vitreous Humor Hyaluronate.*

Vitreous humor was collected from 60 cattle eyes (about 1 liter) and treated in exactly the same way as synovial fluid. The observation of ROBERTSON, ROPES and BAUER (1940) that the viscosity of vitreous humor rapidly decreases was confirmed. This phenomenon is probably due to contamination with hyaluronidase from the ciliary body. The mucin precipitate formed on addition of acetic acid did not resemble synovial fluid mucin, it was more flocculent in appearance. Addition of protein (*c. g.* serum) as recommended by MEYER (*l. c.*) may be advantageous for the complete precipitation of the hyaluronic acid, but was not tried. Potassium hyaluronate prepared from vitreous humor resembled the synovial product, but was somewhat discoloured due to contamination with retinal pigment. Yield: 90 mg (preparation 3, table 1).

#### c) *Umbilical Cord Hyaluronate.*

About 15 umbilical cords were worked up together. They were freed of acetone and finely minced in a meat chopper. Extraction was performed with about 5 volumes of water, with a little toluene added, in the refrigerator for 2 days under occasional stirring. The highly viscous solution was filtered through a double layer of gauze and the filtrate precipitated with 1 per cent acetic acid. The product formed closely resembles that from synovial fluid. Extraction was repeated 3 times in the same way and the combined mucin samples were treated as synovial mucin: Incubation with pancreatin, treatment with trichloroacetic acid, and precipitation with alcoholic potassium acetate. Yield: about 300 mg (preparation 4, table 1). In some cases (preparation

5 and 6) 0.5 gram trypsin (Novo), containing about 15 per cent pure trypsin was used instead of 3 grams of pancreatin. Preparation 7 was made from umbilical cords extracted thoroughly with glacial acetic acid at room temperature as prescribed by MEYER and PALMER (1936). The subsequent treatment was identical with that used for preparation 4.

*Table 1* summarizes the characteristics of the different preparations of potassium hyaluronate. The theoretical nitrogen content

Table 1.

*Characteristics of some potassium hyaluronate preparations.*

Preparation No.	Source of material	Enzymatic treatment	Nitrogen Per cent	Conc. m% <sup>1</sup>	Viscosity
1	Synovial fluid	Pancreatin	2.63	228	12.3
2	— —	none	2.20	232	3.94
3	Vitreous humor	Pancreatin	1.44	248	3.12
4	Umbilical cords	Pancreatin	2.46	240	11.7
5	— —	Trypsin	1.20	244	(3.15) <sup>2</sup>
6	— —	—	1.50	240	(3.80) <sup>2</sup>
7	— —	Acetic acid extraction + Pancreatin	3.22	181	3.50

<sup>1</sup> The term m% (milli percent) is used to designate thousands per cent in stead of the illogical expression: mg%.

<sup>2</sup> These values are not reliable as the preparations displayed a very rapid spontaneous fall in viscosity (cf. text).

calculated from  $(C_{14}H_{20}NO_{11}K)_n$  is 3.36 per cent. It is thus considerably higher than found for most of the preparations. The only one giving nearly the correct N-content is preparation 7. The two batches made from umbilical cords with trypsin digestion were especially interesting in showing less than half the theoretical N-content. Preparations 4 and 5 were made from the same mucin solution, the only difference being the enzymatic treatment (concentrations and volumes were kept identical throughout the whole procedure). The trypsin preparations were further characterised through their great lability. They showed a rapidly decreasing viscosity which really made them quite unfit for hyaluronidase assay. The viscosities entered in *table 1* were measured on solutions of potassium hyaluronate in citrate phosphate buffer, pH 7, which were kept in the refrigerator for about 18 hours, the dissolution being a very slow process. Immediately before measurement the solutions were filtered. A considerable loss of viscosity must be assumed during this period in the case of pre-

parations 5 and 6. BLIX and SNELLMAN (1945) mention that heavy metals may cause depolymerisation of hyaluronic acid. Addition of 8-hydroxyquinoline to the solutions showed a definite greenish colour in the case of preparations made with trypsin, but no reaction with the other solutions. As technical ammonium sulfate, containing traces of iron, is used in the manufacture of commercial

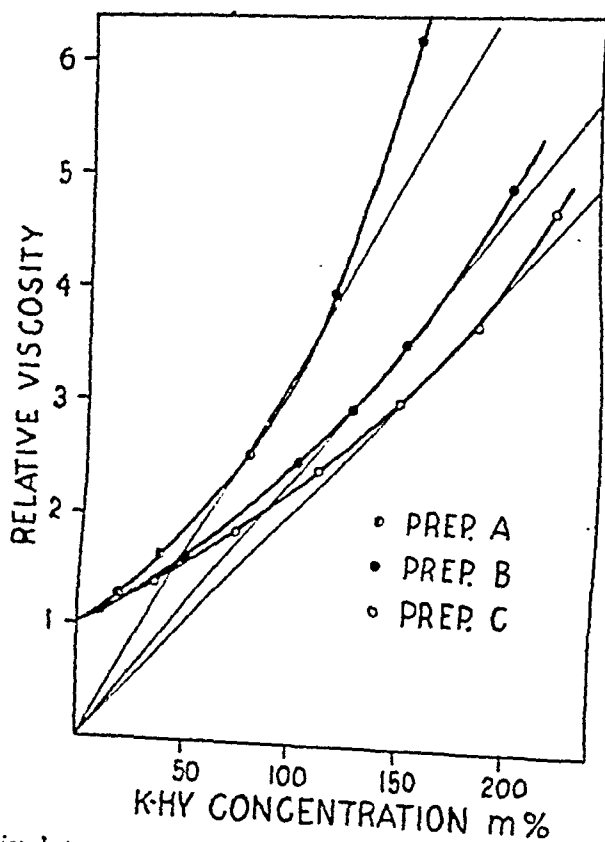


Fig. 1. Relation between viscosity and concentration of solutions containing potassium hyaluronate in citrate-phosphate buffer, pH 7.0.

trypsin, but not in the production of pancreatin it is reasonable to expect a slight contamination with iron. Addition of 0.12 per cent of 8-hydroxyquinoline, which is a strong complex-former, practically abolished the spontaneous decrease in viscosity. This substance can, however, not be used in the assay of hyaluronidase as a considerable inhibition of the enzyme was observed. (Addition of 0.12 per cent of 8-hydroxyquinoline caused 57 per cent inhibition.) The presence of iron can, however, not explain the low nitrogen values. It would appear more probable that some unknown enzyme splitting the nitrogen from the glucosamine residues of the



polysaccharide is present in the trypsin. This problem must, however, await further elucidation. MEYER (*l. c.*) mentions that some preparations of hyaluronic acid contain 50 per cent of inorganic substances, we therefore made ash determinations on preparations 5 and 6. The samples were treated with sulfuric acid and ignited to constant weight. 24 per cent of ash was found in both cases, whereas the theory requires 20.9 per cent as potassium sulfate (9.37 per cent K). Thus inorganic contaminations amount to only a few per cent. Another possibility was the presence of glycogen, but no colour reaction with iodine could be observed.

### Conditions of Hyaluronidase Assay.

Fig. 1 shows the relation between concentration and relative viscosities of three preparations of potassium hyaluronate. The solutions are made up in 0.02 m citrate-phosphate buffer, pH, 7.0 (McILWAIN) containing 0.012 m sodium chloride as used by SWYER and EMMENS (1947). All viscosity and hyaluronidase measurements were made at 34 degrees centigrade in accordance with McCLEAN and HALE (1941). Preparation A from synovial fluid was made from the flocculent precipitate formed after addition of alcoholic potassium acetate (*cf.* above). B was prepared as No. 7 in table 1, and C was made in the usual way from umbilical cords.

For the assay of hyaluronidase activity, estimation of the fall in concentration of hyaluronate, as determined from the curves

Table 2.

*Incubation of various substrate preparations with the same enzyme solution, (bull semen hyaluronidase).*

Preparation No.	Remarks	Substrate conc. m%	Initial viscosity	Activity
1		228	7.0	13.0
1		152	4.0	24.9
2		232	2.7	24.1
3		248	2.6	21.8—24.3
4		240	6.7	16.4—19.2
4		160	3.7	24.6
5	Without enzyme (self splitting)	240		3.4
7	Before the other determinations	211	3.5	5.93
7	After the other determinations	211	3.5	4.37

relating viscosity and concentration, would appear an obvious, though rather cumbersome method. Table 2 shows some results with this method using various substrate preparations.

The experiments were performed in the following way: 3 ml of substrate solution prepared as described above were mixed well with 0.5 ml enzyme solution (made from washed bull spermatozoa) and 3 ml of the mixture were transferred to an Ostwald viscosimeter. The measurement of flowtimes was started immediately. When the logarithm of the hyaluronate concentration, determined as outlined above, is plotted against the time of incubation, straight lines are obtained in all cases, when the splitting of the substrate is below 30 per cent, thus confirming the view that the process is a first order reaction. The enzyme activity is expressed as  $10^{-3}$  times the monomolecular reaction constant (in  $\text{min.}^{-1}$ ), which is simply the slope of the straight line. From the table is apparent that hyaluronate from different sources is split with the same velocity, provided the initial viscosity is not too high (*i. e.* below 4). There is probably also a lower viscosity limit, but this question has not been investigated. Among the experiments performed the only exception appears to be the one where preparation 7 was employed (cf. table 1). This product was split 6 times slower than the others. MEYER (*l. c.*) likewise found the same depolymerisation rate for hyaluronic acid from umbilical cords and synovial fluid, but faster splitting for preparations from malignant tissue and streptococci (167 and 125 per cent respectively). The apparent inhibition of the enzyme in the two cases where more viscous substrate solutions were used may perhaps be a physical phenomenon, though a real inhibition cannot be excluded.

As this method of hyaluronidase measurement involves some calculation, a simpler technique was attempted. As fig. 1 shows, there is approximate proportionality between hyaluronate concentration and the total viscosity when the viscosity interval between 3 and 4 is used. In terms of the generally employed Ostwald viscosimeter this means that the flowtime is a direct measure of the concentration within these limits. When the logarithm of the flowtime is plotted against the time of incubation with enzyme a straight line results, as shown in fig. 2.

The exact course of the concentration-viscosity curve does not influence the slope of the line, when the measurement is performed in the interval where proportionality between flowtime and con-

centration prevails. Very good reaction constants may be obtained in this way, if the splitting does not exceed 20 per cent. From the reaction constant a theoretical "half concentration time" may be calculated according to the formula  $t_{0.5} = \frac{0.693}{k}$ . This is the time which would be needed to reduce the concentration to half the initial value if the first order scheme was followed throughout.

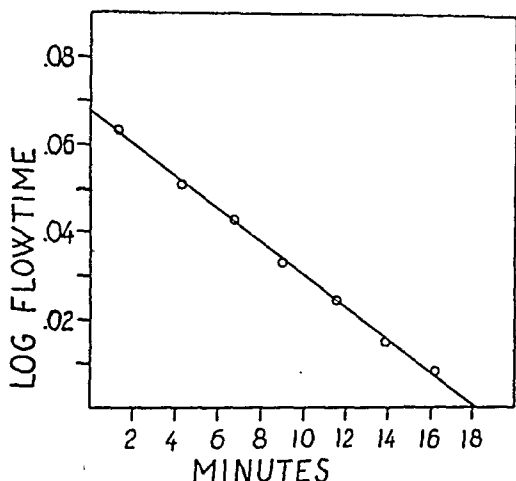


Fig. 2. The logarithm of the flowtime as a function of incubation time.

A similar procedure was employed by WERTHESSEN et. al. (1945), who also used the initial slope of the curve relating the logarithm of the flowtime with the time of incubation as a measure of hyaluronidase activity, but they used low substrate concentrations (10 m%) and consequently were only able to utilise a very short part of the curve (it is stated that the first 4 or 5 points are employed). The accuracy of their method cannot be judged from the data given in the paper.

Table 3.

*Relation between enzyme concentration and reaction constants.*

Enzyme concentration (Arbitrary units)	Substrate concentration	
	163 m%	205 m%
1	105	101
2/3	63	65
1/3	34.4	29.6
1/5		19.2

The viscosimeter used had a flowtime for water of 30.5 seconds.  
The results are expressed as  $10^3$  times the reaction constant ( $\text{min.}^{-1}$ ).

The accuracy of the present method is very satisfactory, double determinations generally agree within 5 per cent and in no case have deviations greater than 10 per cent been observed. As appears from table 3, the activity expressed as reaction constants is proportional to the enzyme concentration within reasonable limits. It is further seen that the substrate concentration need not be exactly reproduced in order to obtain identical results, a fact of some practical importance.

Most of the objections raised in the introduction against the viscosimetric assay of hyaluronidase have been eliminated in the method worked out. 1) The exact time of mixing enzyme and substrate need not be known, as the initial viscosity is not used in the calculation of enzyme activity. 2) The error arising from the decrease in viscosity during the individual flowtime measurements becomes unimportant as the total change in viscosity during the whole experiment does not exceed 20 per cent. 3) Inhibition of the enzyme by reaction products is not encountered.

There is a considerable need for an international, reproducible unit of hyaluronidase. Some workers express their results in terms of a standard enzyme preparation, others use "viscosity reducing units" or "turbidity reducing units", and several other systems for expressing hyaluronidase activity have been employed. Generally the conversion factors relating these various units are not known, so that comparison of results made with different methods of assay is not possible. The definition of an international unit reproducible for all investigators using their own instruments and substrates may perhaps be feasible in terms of the monomolecular reaction constant at specified conditions of temperature, pH, buffer, substrate, etc. As demonstrated in the present work, considerable variations in the substrate with regard to source and method of preparation are tolerated without influencing the enzyme assay. For the purpose of defining a unit it may, however, be safer to stick to one preparation, *c. g.* potassium hyaluronate made from synovial fluid mucin through precipitation with trichloroacetic acid.

The writer wishes to acknowledge the great interest which Prof. KNUD SAND has shown the present investigation. I am also indebted to Mr. CHR. E. JENSEN for valuable assistance.

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### Summary.

1) Some practical and theoretical objections against the viscosimetric assay of hyaluronidase as generally employed have been advanced.

2) A simplified method for the preparation of hyaluronate from vitreous humor, synovial fluid and umbilical cords has been worked out and some properties of the preparations determined.

3) A method for the assay of hyaluronidase using viscosity measurements has been worked out based on the demonstration that the enzymatic depolymerisation follows a monomolecular reaction scheme. The reaction constant under specified conditions is used as a measure of hyaluronidase activity.

4) The feasibility of defining an international unit of hyaluronidase based on measurement of the reaction constant is discussed.

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From the Department of Medical Chemistry, University of  
Uppsala, Sweden.

## On the Occurrence of Bacterial Growth Factors in Liver Extracts.

By

GUNNAR ÅGREN.

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It has been shown in several publications from this laboratory that in the purification of the intrinsic factor activity of the hog's pyloric mucosa this activity closely followed the aminopolypeptidase activity through all the stages of the preparation method (ÅGREN 1944). With small amounts of the highly purified enzyme it was possible to activate the extrinsic factor activity of liver or muscle (ÅGREN and WALDENSTRÖM 1947). The nature of the reaction product was not studied in any detail. However, some of the properties of this product have been investigated by REIMANN and collaborators (REIMANN et al. 1933, 1936) and from their results it is obvious that a relation exists between their reaction product and the anti-pernicious-anemia factor in the liver but differences in the rate of dialysis and thermostability also seemed to occur.

Since a more detailed information about the nature of the reaction product of extrinsic and intrinsic factors might elucidate the properties of extrinsic factor and the enzyme specificity and accordingly the possible identity of intrinsic factor and aminopolypeptidase, a purification of this primary reaction product would be of interest. The convenience of a microbiological method in the testing of fractions from different stages of purification is obvious for all who have tried to purify the anti-pernicious-anemia factor of the liver, but the lack of specificity of some of these

methods is also well known. Accordingly, it was decided first to investigate the possible microbiological growth effect of the already purified and possibly secondary reaction product, *i. e.* the anti-pernicious-anemia factor of the liver.

### Experimental.

*Cultures and inocula.* The organisms tested were *Lactobacillus arabinosus* 17—5 (8014), *Leuconostoc mesenteroides* P-60 (8042), *Streptococcus faecalis* R (8043), *Lactobacillus fermenti* 36 (9338), *Streptococcus lactis* (9790), *Lactobacillus delbrückii* LD5 (9595), *Lactobacillus dextranicum* (8086), *Lactobacillus citrovorum* (8081), *Lactobacillus citrovorum* (8082), *Lactobacillus casei* (7469), *Lactobacillus acidophilus*, *Lactobacillus lactis* 100, *Lactobacillus bifidum*. They were carried by monthly transfers as stab cultures in a basal medium of the following composition: 1 gm. of glucose, 0.5 gm. of Bacto-peptone, 1 gm. of sodium acetate, 100 mg. of  $\text{KH}_2\text{PO}_4$ , 100 mg.  $\text{K}_2\text{HPO}_4$ , 40 mg. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 mg. of  $\text{NaCl}$ , 2 mg. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 mg. of  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$  and 1.5 gm. of agar per 100 ml. of medium at pH 6.8. For the stab cultures of *Lactobacillus lactis* 100, *Lactobacillus acidophilus* and *Lactobacillus bifidum* this medium was supplemented by adding 1 per cent of canned, dried tomato juice, 0.5 per cent of Bacto-peptone, 0.1 per cent of Bacto-yeast and 0.04 per cent of Bacto-liver. All cultures were incubated at  $37^\circ\text{C}$  until good growth occurred in the stab cultures (24 to 48 hrs) and were then refrigerated until next transfer.

Inocula for the assay was prepared by transferring a small amount of growth from a stab culture to a centrifuge tube containing 5 ml. of the same medium but without agar. After incubation for 24 hours the cells of the liquid culture were centrifuged, washed with sterile water or 0.9 per cent sodium chloride solution and diluted to a volume 5 to 50 times greater than that of the medium in which they were grown, the dilution used depending on the amount of growth. 1 drop of suspension was used for each assay tube.

*Procedure.* Assays were carried out in  $18 \times 150$  mm. Pyrex culture tubes and in a total volume of 6 ml. The samples or standards adjusted to pH 6.8 were added in volumes from 0.5 to 3 ml., followed by distilled water to make 3 ml. 3 ml. of the basal medium of HENDERSON and SNELL (1947) adjusted to pH 6.8 was then added. The concentration of medium was that given by HENDERSON and SNELL or in some cases 0.5 times this concentration. In some series the medium of DUNN et al. was used in the same way. Sterilization was accomplished by autoclaving the tubes 5 minutes at 15 pounds pressure. During the incubation in an air incubator for 24 to 72 hours at  $37^\circ$  the growth was followed by turbidimetric analysis in the Klett-Summerson photoelectric colorimeter and simultaneously by direct titration of the lactic acid produced with 0.05 N NaOH. Brom-thymol blue was used as indicator in the acetate containing medium while thymol blue was preferred in the titration of the citrate containing medium. During titration, the content

of each tube was stirred vigorously with a stream of carbon dioxide-free air. The end point of titration was estimated by comparison with tubes containing the buffer mixtures of the medium and the same amount of indicators.

*Preparations tested on the microorganisms.* From the Astra Pharmaceutical Corporation (Sweden) two preparations were obtained which were used in the commercial production of anti-pernicious-anemia preparates, the first more impure with a yield of 20 gm. dry substance from 1 kg. of fresh liver (= Astra A) and the second more purified product with a yield of 0.4 gm. dry material from 1 kg. of fresh liver (= Astra B). From this company a supposedly inactive by-product from the preparation of the active extract was also obtained (= Astra C.). From the Wilson Laboratories (U. S. A.) their fraction L was obtained (= Wilson L.). According to personal information from Dr. KLEIN this fraction should contain only insignificant amounts of the antipernicious-anemia liver factor. A water extract of this preparation was used. The Difco liver extract was prepared from Difco Bacto-Liver according to the description in the Difco Manual. Heptomin is the name of a commercial liver preparation used in this country. To patients with pernicious anemia in relapse 4 ml. is daily administered for 3 to 4 days. Total nitrogen (Kjeldahl) was 1.3 mg. per ml. From the Organon Farmaceutica Corporation (Holland) two preparations were obtained which were supposed to contain in a comparatively impure form the anti-pernicious-anemia liver factor.

As a source of strepogenin activity enzyme digested casein solutions were used. »Aminosol» is the trade name of the preparation described by WRETJÖND 1947 and produced by digestion of casein with trypsin and erepsin followed by dialysis. A similar mixture of low molecular peptides and free amino acids is obtained by catheptic digestion of casein followed by dialysis (ÅGREN 1946). This preparation in the following is named Nutrimin. Since WOOLLEY has stated that strepogenin should be a glutamic acid containing peptide it was decided to try a purification by electrodialysis. This was carried out in an apparatus of the type described by THEORELL and ÅKESON (1943). Determinations with *Lactobacillus casei* demonstrated that in tryptic and catheptic digestions of casein some of the growth activity migrated to the anode compartment. The anode fraction of Aminosol was accordingly also used in some experiments as a source of strepogenin activity which could be suspected to be free from the anti-pernicious-anemia factor. Tested on a patient with pernicious anemia 100 gm. of the catheptic digest was inactive.

*Results.* That the medium of HENDERSON and SNELL is not fully optimal for all strains of lactic acid bacteria used in this investigation is evident since it did not permit good growth and acid production of *Lactobacillus acidophilus*, *Lactobacillus bifidum* and *Lactobacillus lactis* 100. The response of nine of the organisms was further tested by comparing acid production in the original medium



Table 1.

*Lactic acid production on two concentrations of the medium of HENDERSON and SNELL.*

Organism <sup>1</sup>	Medium <sup>2</sup>	Ml. of acid <sup>3</sup>
<i>L. casei</i> (7469) <sup>4</sup> .....	1	9.0 (72)
	0.5	9.8
<i>S. faecalis</i> R (8043) <sup>4</sup> .....	1	18.2 (48)
	0.5	9.2
<i>L. arabinosus</i> 17-5 (8014) <sup>5</sup> .....	1	22.1 (48)
	0.5	10.7
<i>L. citrovorum</i> (8081) <sup>5</sup> .....	1	0 (72)
	0.5	6.6
<i>L. citrovorum</i> (8082) <sup>5</sup> .....	1	11.6 (72)
	0.5	6.0
<i>L. dextranicum</i> (8086) <sup>5</sup> .....	1	0.6 (72)
	0.5	6.0
<i>L. delbrückii</i> LD5 (9595) <sup>4</sup> .....	1	0 (72)
	0.5	10.0
<i>S. lactis</i> (9790) <sup>4</sup> .....	1	14.8 (48)
	0.5	9.5
<i>L. fermenti</i> 36 (9338) <sup>5</sup> .....	1	18.2 (72)
	0.5	11.2

of HENDERSON and SNELL and in a medium containing 0.5 times the concentration of all ingredients in this medium with the exception of Salt C, which was kept at the original concentration. The results of these experiments are given in Table I. In the medium containing one-half of the original amount of solubles the rate of acid production was higher for *Lactobacillus citrovorum* (8081), *Lactobacillus dextranicum* (8086) and *Lactobacillus delbrückii* LD 5 (9595). It seemed probable that the nutritional quality of the medium could be improved in some cases by the addition of a liver extract prepared in the usual fashion from Bacto-Liver (Difco). Each tube of the experimental series was made up to contain 3 mg. or 0.3 mg. of a freshly prepared extract added prior to sterilization. The results of these series of experiments are given in Table 2a and 2b. The growth was followed by direct inspection of the growth in the tubes after 24, 48 and 72 hours. The results of these experiments are given in Table 2a.

<sup>1</sup> The numbers in parenthesis are those given by the American Type Culture Collection. *L. fermenti* was cultivated without citrate in the medium.

<sup>2</sup> The medium of HENDERSON and SNELL used in the same and 0.5 times the concentration stated by these authors.

<sup>3</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are mean values from three tubes and have been corrected for the blank titration. The numbers in parenthesis refer to the incubation times in hours.

<sup>4</sup> Obtained from the American Type Culture Collection.

<sup>5</sup> Obtained through the courtesy of Dr. E. BRAND.

Table 2 a.  
Growth of *Lactobacilli* in synthetic media supplemented with two concentrations of Bacto Liver (Difco).

Organism	Medium <sup>1</sup>	Growth after <sup>2</sup>					
		24 hours		48 hours		72 hours	
		Control	3 mg. liver	Control	3 mg. liver	Control	3 mg. liver
<i>L. casei</i> (743)	0.5	+	+++	++	+++	++	+++
<i>S. faecalis</i> R (8043)	1	+++	+++	+++	+++	+++	+++
<i>L. arabinosus</i> (8014)	1	+++	+++	+++	+++	+++	+++
<i>L. citrovorum</i> (8081)	0.5	+	+++	++	+++	++	+++
<i>L. citrovorum</i> (8082)	1	+++	+++	+++	+++	+++	+++
<i>L. dextranicum</i> (8086)	0.5	++	+++	++	+++	+++	+++
<i>L. delbrueckii</i> (9595)	0.5	++	+++	++	+++	+++	+++
<i>S. lactis</i> (9790)	1	+++	+++	+++	+++	+++	+++
<i>L. fermenti</i> 36 (9398)	1	++	+++	+++	+++	+++	+++
<i>L. mesenteroides</i> P-60	1	+	+	++	+++	+++	+++

<sup>1</sup> The medium of HESPERSON and SNELL used in the same and 0.5 times the concentration stated by these authors.

<sup>2</sup> The growth was estimated by direct inspection and was graded from just detectable growth (+) to luxuriant growth (+++).

Table 2 b.

*Lactic acid production of Lactobacilli in synthetic media supplemented with two concentrations of Bacto Liver (Difco). Incubation times as in Table 1.*

Organism	Medium <sup>1</sup>	Ml. of acid in <sup>2</sup>		
		Control	3 mg. liver	0.3 mg. liver
<i>L. casei</i> (7469) .....	0.5	5.4	5.9	5.6
<i>S. faecalis</i> R (8043) .....	1	9.2	9.7	9.2
<i>L. arabinosus</i> (8014) .....	1	11.3	10.7	10.2
<i>L. citrovorum</i> (8081) .....	0.5	3.9	6.0	5.8
<i>L. citrovorum</i> (8082) .....	1	9.7	7.5	8.0
<i>L. dextranicum</i> (8086) .....	0.5	3.4	3.8	3.4
<i>L. delbrückii</i> LD5 (9595) ..	0.5	5.5	5.8	5.7
<i>S. lactis</i> (9790) .....	1	8.1	8.4	7.2
<i>L. fermenti</i> 36 (9338) .....	1	8.3	9.6	9.0
<i>L. mesenteroides</i> P-60 .....	1	10.4	10.5	10.2

At the end of the incubation time the lactic acid was titrated in the usual way. The results of these experiments are recorded in Table 2b. From the observations given in Table 2a it was concluded that a more pronounced difference in growth between controls and liver tubes only could be found in the series with *Lactobacillus casei* (7469) and *Lactobacillus citrovorum* (8081). For *Lactobacillus casei* this difference had disappeared after 72 hours of incubation while *Lactobacillus citrovorum* still showed a considerable difference in growth and acid production between controls and liver tubes (Table 2a and 2b) after this time. A slight growth effect of the liver extract was also found in the series with *Lactobacillus fermenti* 36.

The growth effect of the Difco liver extract in the medium of HENDERSON and SNELL in some way reminded of that obtained by WOOLLEY and SPRINCE in their medium with strepogenin. These authors used as test organisms *Lactobacillus casei* and *Streptococcus lactis*, and they demonstrated the presence of strepogenin in certain liver extracts. In the medium of HENDERSON and SNELL comparatively large amounts of the Difco liver extract did not influence the growth of *Streptococcus lactis* (9790) and only slightly the growth of *Streptococcus lactis* R (8043) (Table 2a). This discrepancy possibly could be explained by different composition of the two media. Accordingly, the next step was to test the effect of more purified extracts of liver and strepogenin on the growth of *Lactobacillus citrovorum* (8081) and *Lactobacillus casei*.

<sup>1</sup> The medium of HENDERSON and SNELL used in the same and 0.5 times the concentration stated by these authors.

<sup>2</sup> Given as ml. of 0.05 N NaOH to titrate 3 ml. of final solution. The values are the mean of three tubes and have been corrected for the blank titrations.

Table 3.

*Growth of Lactobacillus citrovorum* (8081) in synthetic medium supplemented with different anti-pernicious-anemia and strepogenin extracts. Time of incubation 24, 48 and 72 hours.

Preparations <sup>1</sup>	ml. of acid produced with the following amounts of substances added per tube <sup>2</sup>														
	0 mg.			4.8 mg.			0.48 mg.			0.0048 mg.			0.000048 mg.		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
Astra A .....	4.5	10.8	11.0	10.5	11.5	11.4	7.6	10.8	11.1	5.4	9.3	9.6	5.4	7.1	—
Astra B .....	4.5	10.8	11.0	10.4	11.5	11.6	7.8	10.2	11.0	6.8	9.6	10.2	5.8	—	—
Wilson L .....	4.2	5.7	—	11.6	11.2	—	9.8	10.5	—	5.8	7.2	—	5.3	6.8	—
Difco liver .....	4.2	5.8	—	10.3	11.5	—	10.3	11.5	—	5.9	7.0	—	5.7	7.0	—
Aminosol .....	4.8	7.5	—	8.0	10.0	—	4.6	7.3	—	—	—	—	—	—	—
Aminosol anode ...	4.8	7.6	—	8.2	10.3	—	5.4	7.8	—	4.2	6.5	—	—	—	—
Nutrimin .....	4.9	7.5	—	9.2	9.9	—	6.8	8.3	—	4.2	7.3	—	—	—	—
Growth response simultaneously registered with photoelectric colorimeter <sup>3</sup>															
Astra A .....	82	108	—	358	393	—	309	327	—	130	152	—	117	139	—
Astra B .....	85	109	—	242	291	—	303	331	—	127	153	—	118	142	—
Wilson L .....	81	110	—	355	388	—	233	241	—	115	130	—	105	125	—
Difco liver .....	81	105	—	396	426	—	313	334	—	107	129	—	109	131	—
Aminosol .....	95	134	—	188	227	—	94	121	—	—	—	—	—	—	—
Aminosol anode ...	98	134	—	149	170	—	101	132	—	89	110	—	—	—	—
Nutrimin .....	97	135	—	210	250	—	133	157	—	89	123	—	—	—	—

*Results with Lactobacillus citrovorum.* Typical growth responses of *Lactobacillus citrovorum* (8081) to graded amounts of different commercial liver preparations and strepogenin extracts are given in Table 3. The growth was followed simultaneously by titrations and photoelectric readings. With both techniques additional growth could be registered when strepogenin containing extracts as Aminosol or Nutrimin were added to the medium. A certain amount of accumulation of the growth activity of Aminosol was also found in the anode fraction after electro-dialysis of the preparation. On the other hand the growth responses were more conspicuous with the liver extracts. Significant differences could not be obtained between the values of the series with the Wilson liver fraction L and the values of the series with

<sup>1</sup> Described in the text.

<sup>2</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are mean values from three tubes and have been corrected for the blank titrations.

<sup>3</sup> Given as scale readings on the Klett-Summerson photoelectric colorimeter. The reading 0 corresponds to 100 per cent transmission and the values have been corrected for the blank values.

the Astra preparations. The former preparation was stated to contain only insignificant amounts of the anti-pernicious-anemia factor while the latter were supposed to be active in this respect. The possibility remained that *Lactobacillus citrovorum* reacted only to the strepogenin activity present in all extracts. A further testing of the specificity of the microorganism was accordingly necessary and attempts were made to separate the two factors by differences in thermo-stability. WOOLLEY and SPRINCE stated that strepogenin present in tissue extracts and tryptic digests of pure proteins were unaffected by autoclaving in the basal medium. On the other hand it is difficult to obtain any accurate data for the thermostability of the anti-pernicious-anemia factor in liver during the heating conditions routinely used in microbiological procedures. Accordingly, the influence of different autoclaving times on the growth effect of preparations containing strepogenin and the blood stimulating factor were investigated. Graded amounts of the preparations to be assayed were added to series of tubes in the usual way and autoclaved in the medium for 5 minutes. The time taken to obtain the 15 pounds of pressure in the autoclave was 5 minutes and the cooling to room temperature was finished after 2 minutes. In addition, parallel assays were performed in which solutions of the substances sterilized by filtration were added to the basal medium after the latter had been autoclaved. By filtrating comparatively large volumes through filters of small diameters nitrogen losses were reduced to less than 1 per cent. Typical responses in growth from these series are recorded in Table 4. A comparison of these data clearly demonstrates that an autoclaving time of 5 minutes did not inactivate the growth activity of the liver extracts or of the enzyme digests of casein. Accordingly, a similar series of experiments were carried out where the preparations to be assayed were autoclaved in the medium for 15 minutes or added aseptically to the autoclaved medium after sterilization by filtration. Typical responses in growth from some series are shown by the data in Table 5. From the figures in the Table it is obvious that the growth activity of the liver extract containing the antipernicious anemia factor (Astra A) was not influenced by autoclaving for 15 minutes at 15 pounds pressure. The same heat-stability was demonstrated by the Wilson fraction L, supposed to contain only strepogenin activity. In a separate experiment it could be shown that the commercial liver extract Heptomin, which in this country is used as an injection prepara-

Table 1.

Growth of *Lactobacillus catenarium* (SOSI) after adding extracts containing the anti-pernicious-anemia liver factor and (or) streptogenin to the basal medium of HENRIKSEN and SÆVIK followed by autoclaving for 5 minutes at 15 pounds pressure.

Preparation <sup>1</sup>	Photoelectric readings after 24 and 48 hours of incubation. <sup>2</sup> The amounts of substances added per tube were the following							
	0 mg.		4.3 mg.		0.13 mg.		0.0013 mg.	
	24	48	24	48	24	48	24	48
Nutrimin .....	7	(7) 10	(15) 128	(112) 165	(179) 15	(17) 21	(122) 7	(8) 12
Aminocol .....	7	(7) 12	(15) 103	(120) 113	(196) 14	(9) 35	(77) 8	(10) 30
Astra A .....	3	(6) 4	(93) 330	(345) 465	(376) 270	(266) 355	(307) 3	(1) 13
Wilson L .....	4	(6) 4	(94) 365	(355) 413	(395) 111	(86) 210	(120) 7	(12) 15
ML. of acid produced in the same series <sup>3</sup>								
Nutrimin .....	0	(0) 0.3	(0.3) 4.4	(5.1) 8.4	(8.4) 0.5	(0.4) 1.1	(2.8) 0	(0) 0.3
Aminocol .....	0	(0) 0.3	(0.3) 2.7	(0.5) 7.1	(4.8) 0.2	(0) 0.9	(0.6) 0	(0) 0.3
Astra A .....	0	(0) 0	(0) 8.4	(9.9) 10.9	(11.1) 7.2	(6.0) 10.0	(9.8) 0	(0) 0.5
Wilson L .....	0	(0) 0	(0) 9.3	(8.4) 9.9	(10.1) 2.7	(2.6) 8.9	(9.1) 0	(0) 0.6

<sup>1</sup> Described in the text.

<sup>2</sup> Given as scale readings on the Klett-Summerson photoelectric colorimeter. The reading 0 corresponds to 100 per cent transmission and the values have been corrected for the blank values. The values in parenthesis are those obtained by aseptical addition of the filter-sterilized extracts to the tubes.

<sup>3</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are the means from three tubes and have been corrected for the blank titrations. The values in parenthesis are those obtained by aseptical addition of the filter-sterilized extracts to the tubes.

Table 5.

*Growth of Lactobacillus lactis (3081) after adding extracts containing the anti-pernicious-anemia liver factor and (or) streptogenin to the basal medium of HENDERSON and SNELL followed by autoclavation for 15 minutes at 15 pounds pressure.*

Preparations <sup>1</sup>	Photoelectric readings after 24 and 48 hours of incubation. <sup>2</sup> The amounts of substance added per tube were the following							
	0 mg.		4.8 mg.		0.48 mg.		0.0048 mg.	
	24	48	24	48	24	48	24	48
Astra A.....	13	(14) 25	(30) 465	(480) 490	(480) 330	(320) 350	(345) 14	(20) 18
Wilson L.....	14	(13) 26	(33) 440	(430) 450	(430) 226	(225) 350	(250) 17	(14) 40
ml. of acid <sup>3</sup> produced in the same time <sup>3</sup>								
Astra A.....	0.3	(0.6) 0.5	(0.6) 10.0	(10.4) 11.1	(11.1) 8.6	(9.0) 10.4	(10.5) 0.4	(0.6) 0.8
Wilson L.....	0.3	(0.6) 0.5	(0.7) 10.2	(10.2) 11.5	(10.5) 7.6	(8.0) 10.4	(9.7) 0.4	(0.6) 0.5

<sup>1</sup> Described in the text.

<sup>2</sup> Given as scale readings on the Klett-Summerson photoelectric colorimeter. The reading 0 corresponds to 100 per cent transmission. The values in parenthesis are those obtained by aseptical addition of the filter-sterilized extracts to the autoclaved medium and have been corrected like all values for the blank values.

<sup>3</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are mean values from three tubes and have been corrected for the blank titrations.

tion in the treatment of pernicious anemia could be autoclaved for 30 minutes at 15 pounds pressure and still seemed to contain full activity when tested on a patient with pernicious anemia. The data from this case will be published elsewhere. From all these experiments it was concluded that both the antipernicious anemia factor of liver and strepogenin was very heat stable. Differences in this property could not advantageously be used to decide if the growth activity of the tested liver preparations depended on contamination with strepogenin or if the anti-pernicious-anemia factor of the extracts stimulated the growth of the microorganism.

It has previously been shown that the growth activity of an enzymatic casein hydrolysate could be separated into active fractions by electrodialysis (cf. Table 3). The same method could be expected to separate strepogenin from the anti-pernicious-anemia factor in liver extracts if the latter stayed in the middle compartment or moved to the cathode while the glutamic acid containing strepogenin was accumulated in the anode fraction. Since Heptomin is a reliable commercial liver extract, 200 ml. of the preparation were resolved into basic, neutral and acidic fractions by electrodialysis employing the device described by THEORELL and ÅKESON. An analysis in the desalting apparatus of CONSDEN, GORDON and MARTIN (1947) showed that the preparation only contained traces of inorganic salts. Accordingly, it could be expected that the fractions moving into the anode and cathode compartments would not be destroyed by the influence of inorganic ions moving simultaneously into the compartments. The following distribution of nitrogen calculated in percentage of total nitrogen was found:

Cathode fraction .....	24.4 %
Middle fraction .....	61.5 %
Anode fraction .....	16.1 %
Recovered nitrogen .....	102.0 %

At the end of the electrodialysis the pH of the different fractions were the following:

Cathode fraction .....	pH 7.9
Middle fraction .....	pH 5.5
Anode fraction .....	pH 3.5

For the microbiological tests all fractions were neutralized to pH 6.8. Typical growth responses of the three fractions are given in Table 6. When the nitrogen was separated in three fractions



Table 6.

Growth of *Lactobacillus citrovorum* (8081) after aseptical addition of fractions from electrodyalized Heptomin to the autoclaved basal medium of HENDERSON and SNELL. The amounts added are expressed as mg. of total nitrogen. Photoelectric readings after 24 and 48 hours of incubation.

Anode fraction						Middle fraction						Cathode fraction					
0		0.21 mg.		0.021 mg.		0		0.8 mg.		0.08 mg.		0		0.32 mg.		0.032 mg.	
24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
6	11	60	230	16	50	6	12	64	121	19	28	6	11	27	55	20	31
Milliliters of acid simultaneously produced <sup>2</sup>																	
0.2	0.4	1.0	5.3	0.3	0.8	0.2	0.4	1.0	4.9	0.5	1.0	0.2	0.3	0.5	1.1	0.4	0.8

by electrodialysis the growth effect was also spread in all the fractions. It could be suspected that by this means it would be possible to facilitate the use of *Lactobacillus citrovorum* (8081) to test the anti-pernicious-anemia liver factor also in an impure state. If it could be demonstrated that the anode fraction was inactive in man, and any of the other two fractions from the electrodialysis, or both, were active when given to patients with pernicious anemia this condition would be fulfilled. The substance moving into the anode compartment could be suspected to be related to strepogenin and accordingly inactive as a blood stimulating factor. The results with testing the three fractions on patients with pernicious anemia have recently been performed and shown that the anode fraction was inactive. The activity chiefly remained in the middle fraction. These cases will be reported elsewhere.

*Results with Lactobacillus casei.* In the meanwhile the results obtained when investigating the growth effect of liver extracts and enzyme hydrolysates of casein on some other micro-organisms will be reported. Evidence has previously been given in Table 2a that the addition of Bacto-liver to the medium of HENDERSON and SNELL increased the growth of *Lactobacillus casei* when measured after 24 hours of incubation. After 72 hours of incubation all tubes, whether supplemented or not, showed maximal growth and acid production. A very similar effect was

<sup>1</sup> Given as scale readings on the Klett-Summerson photoelectric colorimeter. Mean values from three tubes corrected for blank values.

<sup>2</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. Mean values from three tubes corrected for the blank titrations.

obtained by SPRINCE and WOOLLEY with *Lactobacillus casei* when they added strepogenin containing material to their synthetic medium. On the other side *Lactobacillus citrovorum* (8081) still showed an extra growth effect after 72 hours of incubation with the same liver extract (Table 2a). The possibility therefore existed that *Lactobacillus casei* and *Lactobacillus citrovorum* somehow reacted differently for the anti-pernicious-anemia factor and strepogenin. The series reported in Table 4a were performed with *Lactobacillus casei* in the same way as those given in Table 4 to permit a comparison with *Lactobacillus citrovorum*. This comparison gives some interesting results. In both series it is clear that the growth factors of the enzyme hydrolysates and the liver extracts are heat stable during the experimental conditions. Comparing the enzyme hydrolysates (Aminosol and Nutrimin) with the liver extracts (Astra and Wilson) the main picture obtained from Table 4 is the following. Both with regard to turbidimetric and titrimetric analysis the liver extracts are the more potent for *Lactobacillus citrovorum*. This differences is quite obvious both in the tubes supplemented with 4.8 and 0.48 mg. of substances and after 24 and 48 hours of incubation. The two enzyme hydrolysates have about the same activity. Of the liver extracts the Astra preparation is the more potent which can be seen from the tubes supplemented with 0.48 mg. of substance and incubated for 24 hours.

In contrast to these results it is clear from the 24 hours figures in Table 4a that the enzyme hydrolysates were the more potent when tested with *Lactobacillus casei*. This fact is most easily observed in the series where 0.48 and 0.0048 mg. of substance had been added to the tubes. The last amount of material was inactive when tested with *Lactobacillus citrovorum* and 0.48 mg. of substance was almost inactive. Of the two liver extracts the Wilson preparation was the more active both with regard to turbidimetric and titrimetric analysis. This fact can be read from a comparison in Table 4a of the figures obtained with the tubes supplemented with 0.48 mg. of substance and incubated for 24 hours. Accordingly it could be supposed that both microorganisms responded to the addition of material containing strepogenin (Aminosol, Nutrimin, Wilson L) and the anti-pernicious-anemia factor of liver, but there was a quantitative difference between the responses of the two bacteria. *Lactobacillus casei* seemed to react stronger for the first type of material, *Lactobacillus citrovorum* for the second. This impression was strengthened when the electrodyalyzed frac-

Table 4 a.  
*Growth of Lactobacillus casei in the medium of HENDERSON and SNELL during the same experimental conditions as given in Table 4.*

Preparations	Photoelectric readings after 24 and 28 hours of incubation. The amounts of substances added per tube were the following									
	0		4.8 mg.		0.48 mg.		0.0048 mg.			
	24	48	24	48	24	48	24	48		
Nutrimin . . . . .	37	(35) 345	(305) 302	(300) 338	(360) 255	(260) 326	(350) 64	(75) 332	(330)	
Aminosol . . . . .	39	(35) 345	(301) 296	(292) 350	(360) 250	(252) 335	(355) 93	(87) 360	(340)	
Aminosol anode . . . . .	44	— 330	— 242	— 340	— 146	— 320	— 137	— 330	—	
Astra A . . . . .	24	(19) 320	(275) 204	(153) 424	(370) 108	(68) 388	(316) 22	(30) 330	(289)	
Wilson L . . . . .	22	(11) 325	(274) 251	(185) 415	(395) 160	(100) 372	(330) 14	(20) 324	(270)	
Milliliters of acid simultaneously produced										
Nutrimin . . . . .	0.6	(0.5) 9.9	(9.1) 8.1	(8.0) 10.0	(10.1) 7.0	(6.0) 10.2	(9.9) 1.0	(1.1) 10.1	(9.7)	
Aminosol . . . . .	0.6	(0.7) 9.7	(9.3) 8.0	(7.9) 9.9	(10.1) 7.0	(5.9) 10.1	(9.9) 1.3	(1.5) 10.2	(10.1)	
Aminosol anode . . . . .	1.0	— 9.8	— 5.9	— 10.3	— 3.8	— 10.2	— 3.1	— 9.6	—	
Astra A . . . . .	0.2	(0.3) 8.6	(8.0) 4.2	(2.6) 10.6	(10.2) 1.8	(1.0) 10.4	(9.0) 0.3	(0.4) 9.2	(8.3)	
Wilson L . . . . .	0.2	(0.2) 8.5	(7.8) 4.5	(4.0) 9.8	(9.7) 2.9	(2.8) 10.5	(9.9) 0.2	(0.7) 8.9	(7.5)	

tions of Heptomin were tested on *Lactobacillus casei* in the same way as previously performed with *Lactobacillus citrovorum*. Typical growth responses from these experiments are given in Table 6a. The figures from the 24 hours series are of considerable interest when compared with the corresponding data in Table 6. To facilitate a comparison the two sets of figures are collected in Table 7. One point of interest which can be observed in the Table is that the cathode and middle fractions have about the same activity for both microorganisms. However, with the anode fraction considerably higher growth figures were obtained with *Lactobacillus casei*. The fact that the neutral and basic fractions also stimulated the growth of *Lactobacillus casei* are of special interest with regard to the strepogenin question. This peptide contains according to WOOLLEY glutamic acid and accordingly it would seem likely that strepogenin moved to the anode compartment at the electrodialysis. The highly purified liver preparation Heptomin obviously also contains growth factors for *Lactobacillus casei* of more neutral or basic nature. The cathode fraction contained a biuret positive substance and on a paper chromatogram several ninhydrin positive spots could be observed.

*Results with Lactobacillus acidophilus.* In the search for other microorganisms giving additional growth when the medium was supplemented with enzyme hydrolysates or liver extracts one of several investigated strains of *Lactobacillus acidophilus* showed some response. The microorganism showed a poor growth on the two concentrations of the HENDERSON and SNELL medium described in Table 1. Better results were obtained by exchanging sodium citrate for sodium acetate of the same concentration. Additional acid production was obtained by supplementing the tubes with 4.8 mg. of Bacto-liver (Difco) or (and) 15 mg. of a tomato juice preparation. This was obtained by centrifugation of canned tomato juice followed by lyophilization of the centrifugate. The medium of DUNN et al. (1947) was investigated in the same way. Typical growth figures are recorded in Table 8. From the data in the Table there seems to be a slight superiority of the acetate over the citrate buffers. Additional growth in the two media was observed after a supplement with liver. Tomato juice also contained growth-promoting activity for this lactobacillus. The medium of DUNN et al. was used in the experiments with this microorganism. The series reported in Table 4b were performed in the same way as those described in Table 4 with

Table 6 a. (Table 6 continued).  
Growth of *Lactobacillus casei* during the same experimental conditions as given in Table 6.

*Grown of Lactococcus*

*Table b.*

Anode fraction						Middle fraction						Cathode fraction														
0			0.21 mg.			0.021 mg.			0			0.8 mg.			0.08 mg.			0			0.32 mg.			0.032 mg.		
24	48		24	48		24	48		24	48		24	48		24	48		24	48		24	48		24	48	
13 <sup>1</sup>	193		280	385	133	298	15	192	51	363	27	300	14	195	46	375	19	265								

Milliliters of acid simultaneously produced<sup>2</sup>

0.3	6.7	15.7	10.1	2.0	9.6	0.3	6.7	0.8	9.8	0.4	9.2	0.3	6.7	0.7	9.4	0.4	8.3
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Table 7.

Growth of *Lactobacillus casei* (7469) and *Lactobacillus citrovorum* (8081) in basal medium with addition of fractions from electrolyzed Heptamin. Incubation time 24 hours. Data from Tables 6 and 6 a.

Method of analysis	Anode fraction			Middle fraction			Cathode fraction		
	0.21 mg.	0.021 mg.		0.8 mg.	0.08 mg.		0.32 mg.		
Turbidimetric <sup>1</sup> .....	(7469) (8081)	(7469) (8081)	(7469) (8081)	(7469) (8081)	(7469) (8081)	(7469) (8081)	(7469) (8081)	(7469) (8081)	(7469) (8081)
Titrimetric values <sup>2</sup> .....	280 60 133 1.0 2.0 5.7	16 51 64 1.0 0.4 0.7	19 27 19 0.4 0.5 0.4	27 19 46 0.7 0.4 0.4	27 19 46 0.7 0.4 0.4	27 19 46 0.7 0.4 0.4	27 19 46 0.7 0.4 0.4	27 19 46 0.7 0.4 0.4	27 19 46 0.7 0.4 0.4

<sup>1</sup> Given as scale readings on the Klett-Summerson colorimeter. Mean values from three tubes corrected for blank readings.

<sup>2</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solutions. Mean values from three tubes corrected for blank titrations.

Table 8.

Growth of *Lactobacillus acidophilus* in synthetic media supplemented with Bacto liver and tomato juice extract. Incubation times 24, 48 and 72 hours.

Media	Ml. of acid in medium supplemented per tube with <sup>1</sup>								
	Control			4.8 mg. of liver			4.8 mg. of liver + 15 mg. of tomato extract		
	24	48	72	24	48	72	24	48	72
HENDERSON and SNELL (A) ...	0.4	0.5	—	—	—	—	1.0	1.2	—
HENDERSON and SNELL 0.5 times the concentration in A (B) ...	0.2	0.3	—	—	—	—	1.0	1.2	—
A without citrate + acetate ...	1.2	2.0	1.3	1.4	4.6	4.3	—	—	—
B without citrate + acetate ...	0.6	0.8	0.7	1.2	3.0	2.6	3.4	4.8	—
DUNN et al. ....	1.3	1.8	3.1	2.2	6.0	3.1	5.6	7.1	—

<sup>1</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are mean values from three tubes and have been corrected for the blank values.

Table 4 b. (Table 4 continued).

Growth of *Lactobacillus acidophilus* during the same experimental conditions as given in Table 4, with the exception of the basal medium in this series being that of DUNN et al.

Preparations	Ml. of acid produced with the following amounts of substance added per tube <sup>1</sup>							
	0 mg.		4.8 mg.		0.48 mg.		0.0048 mg.	
	24	48	24	48	24	48	24	48
Aminosol .....	0.4	0.9	0.5	2.8	0.4	0.9	0.4	0.6
Astra A .....	0.2	0.4	0.5	3.0	0.2	0.7	0.2	0.4
Astra B .....	0.6	1.6	4.0	4.8	1.8	3.0	0.7	1.4
Wilson L .....	0.2	0.4	0.5	1.4	0.3	0.5	0.2	0.5

<sup>1</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution.

*Lactobacillus citrovorum* and in Table 4a with *Lactobacillus casei* to permit a comparison of the growth effects of the tested enzyme hydrolysates and liver extracts. Only acidimetric analyses were carried out. Like the two last mentioned microorganisms *Lactobacillus acidophilus* also increased in growth both when streptogenin or anti-pernicious-anemia factor containing material was added to the medium. As mentioned before *Lactobacillus casei* seemed to produce more acid on streptogenin containing material (Aminosol,

Nutrimin) while *Lactobacillus citrovorum* produced more acid on material containing the anti-pernicious-anemia factor of liver (Astra). *Lactobacillus acidophilus* seemed to produce about the same amount of acid when either of the two types of material was added to the basal medium. One interesting result is possibly the fact that the preparation Astra B seemed to be more active than Astra A. Astra B contained the anti-pernicious-anemia factor in a more pure form than Astra A. A parallel series to the experiments described in Table 5 was also carried out with this microorganism. Typical results are recorded in Table 5a. The growth figures were low when compared with the results obtained with the two earlier tested microorganisms both when turbidimetric and titrimetric analysis were used as indicators of the growth. It is possible that the medium of DUNN et al. although supplemented with liver extract or enzyme hydrolysate did not permit optimal growth of *Lactobacillus acidophilus*. Much better growth responses were observed when the microorganism was cultured on milk. Tomato juice may also contain additional growth factors (Table 8).

*Results with Lactobacillus lactis 100.* At this stage of the investigation the preliminary communication of SHORB (1947) appeared where it was shown that this microorganism cultured on a non-specified basal medium required the presence of a heat stable growth factor present in liver extracts but not in casein hydrolysates. Consequently it was of interest to compare the growth properties of this microorganism with the previously investigated. Different variations of the medium of HENDERSON and SNELL and that of DUNN et al. were analyzed in the same manner as carried out on *Lactobacillus acidophilus* (Table 8). The same liver and tomato juice extracts were used. Some of the results are summarized in Table 9. This survey showed that the only two media which could be used with some success were the HENDERSON and SNELL medium with sodium citrate exchanged for sodium acetate or the medium of DUNN et al. The latter medium is the more expensive of the two and a further comparison between the two media was therefore carried out using the preparations Astra A and B. The results are given in Table 10. The acid production was higher in the series incubated with the medium of DUNN et al., but a considerable growth response was also obtained on the less expensive medium of HENDERSON and SNELL which accordingly was used in the further experiments with this microorganism. The use of the latter medium also facilitated a compari-

Table 5 a. (Table 5 continued).

*Growth of Lactobacillus acidophilus during the same experimental conditions as given in Table 5. Medium of DUNN et al.*

Preparations	Photoelectric readings after 24 and 48 hours of incubation. The amounts of substances added per tube were the following									
	0		4.8 mg.		0.18 mg.		0.0018 mg.			
	24	48	24	48	24	48	24	48	24	48
Nutrimin .....	69	—	92	—	114	—	78	—	96	—
Aminosol .....	68	—	103	—	126	—	80	—	108	—
Astra A .....	58	(92)	105	(92)	120	(105)	82	(70)	118	(90)
Wilson L .....	57	(92)	93	(97)	131	(129)	82	(95)	109	(105)
Milliliters of acid simultaneously produced										
Nutrimin .....	1.5	—	1.6	—	2.1	—	1.6	—	1.7	—
Aminosol .....	1.5	—	1.5	—	2.1	—	1.8	—	2.2	—
Astra A .....	1.6	(1.5)	2.0	(1.6)	4.4	(3.9)	5.6	(4.0)	2.8	(2.1)
Wilson L .....	1.6	(1.6)	2.2	(1.6)	2.4	(2.1)	3.7	(2.8)	1.9	(1.4)



Table 9.

*Growth of Lactobacillus lactis 100 in synthetic media supplemented with liver and tomato juice extract. Incubation time 24, 48 and 72 hours.*

Media	Ml. of acid produced in medium supplemented per tube with <sup>1</sup>					
	Control			4.8 mg. of liver and 15 mg. of tomato extract		
	24	48	72	24	48	72
HENDERSON and SNELL (A) .....	0	0.6	1.2	0	1.6	2.0
HENDERSON and SNELL 0.5 times the concentration in A (B) .....	0	0.2	0.4	0	0.6	1.2
A with citrate exchanged for acetate..	0	0.6	1.0	1.0	6.7	10.6
B with citrate exchanged for acetate..	0	0	0.4	0	2.2	3.2
DUNN et al. ....	0	6.0	13.5	0	14.0	16.0

<sup>1</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are mean values of three tubes and have been corrected for the blank values.

Table 10.

*Growth of Lactobacillus lactis 100 in synthetic media supplemented with liver and tomato juice extract. Incubation times 24, 48 and 72 hours.*

Synthetic media and liver preparations	Ml. of acid produced <sup>1</sup> in medium supplemented per tube with											
	Control			4.8 mg. of liver			0.48 mg. of liver			0.0048 mg. of liver		
	24	48	72	24	48	72	24	48	72	24	48	72
HENDERSON and SNELL with citrate exchanged for acetate and supplemented with:												
Astra A .....	1.0	8.4	10.0	1.0	10.6	11.0	0.8	10.2	10.8	0.4	10.2	10.7
Astra A + 15 mg. of tomato juice extract .....	1.1	8.6	10.5	1.0	8.0	—	0.9	7.5	—	0.7	7.1	—
Astra B .....	1.0	8.5	10.0	1.1	10.5	11.0	1.0	10.9	10.8	0.8	10.2	11.0
Astra B + 15 mg. of tomato juice ..	1.5	8.9	10.2	1.4	7.8	—	0.8	7.4	—	0.7	7.1	—
DUNN et al. supplemented with:												
Astra A .....	0	6.0	13.5	0	10.0	15.0	0	4.6	13.1	0	6.8	14.2
Astra B .....	0	6.1	13.6	0	10.8	15.0	0	6.9	12.6	0	7.0	12.1

<sup>1</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are the mean values from three tubes and have been corrected for the blank titrations.

son with the results obtained with *Lactobacillus citrovorum* and *Lactobacillus casei*. On the other hand the possibility existed that the medium of DUNN et al. would be preferable for quantitative determinations of the anti-pernicious-anemia factor. Tomato juice extract did not further increase the acid production of *Lactobacillus lactis* when the Astra preparations were present in the medium of HENDERSON and SNELL or DUNN et al. This extra growth effect of tomato juice was only observed in experiments when Bacto-liver was present in the medium. The experiments recorded in Table 4c were carried out during the same experimental conditions as those described in Table 4 in order to permit a comparison with the other microorganisms.

Obviously *Lactobacillus lactis* also gave an extra growth response when strepogenin or anti-pernicious-anemia factor containing material was added to the basal medium. Of the strepogenin preparations tested the Wilson liver fraction L was the most active. The same results were obtained with *Lactobacillus citrovorum*. In fact this preparation seemed to be active in higher dilutions than the Astra preparation containing the antianemia factor, while one of the Organon liver preparations (B) also containing the factor in purified form was perhaps active in the same dilutions as the Wilson preparation. The thermostability of the growth factors present in the enzyme hydrolysates and liver extracts had previously been tested in experiments where the preparations to be assayed were autoclaved in the medium for 15 minutes at 15 pounds pressure (Table 5 and 5a). Inactivation was not observed. A similar series of experiments were also carried out with *Lactobacillus lactis*. The preparations were now autoclaved for 30 minutes at the same pressure and added to the medium autoclaved as usually for 5 minutes at the same pressure. As a control filter-sterilized solutions were added to the autoclaved medium. Typical results are recorded in Table 11. From the figures of this table it is obvious that the two preparations retained their total activity after the heating. The strepogenin activity of Aminosol must be considered as highly thermostable. The same degree of thermostability is a characteristic property of the anti-pernicious-anemia factor in liver as demonstrated on a patient with pernicious anemia. The figures for the Astra preparation in Table 11 do not contradict this result. Accordingly, differences in heat stability could not advantageously be used to decide how much of the growth activity of the tested liver extracts depended on contamination with stre-

Table 4 c. (Table 4 continued).

*Growth of Lactobacillus lactis 100 in the medium of HENDERSON and SNELL during the same experimental conditions as given in Table 4. Incubation time 24 and 48 hours.*

Preparations	Photoelectric readings, the amounts of substances added per tube were											
	0			4.8 mg.			0.48 mg.			0.0048 mg.		
	24	48		24	48		24	48		24	48	
Nutrimin .....	26	(32) 42	(37) 84	(212) 153	(326) 26	(39) 33	(73) 22	(37) 36	(36)			
Aminosol .....	60	— 85	— 200	— 380	— 65	— 82	— 63	— 79	—			
Astra A .....	46	(45) 66	(68) 120	(276) 263	(380) 54	(67) 92	(194) 40	(61) 50	(81)			
Astra C .....	53	(55) 60	(58) 55	(65) 70	(70) 60	(70) 70	(75) 55	(70) 60	(70)			
Wilson L .....	65	(70) 89	(93) 60	(371) 258	(498) 65	(112) 178	(400) 60	(115) 75	(178)			
Organon A .....	52	(58) 54	(55) 120	(115) 150	(115) 81	(70) 107	(70) 55	(60) 72	(58)			
Organon B .....	8	(10) 12	(20) 209	(218) 335	(340) 21	(31) 133	(106) 25	(26) 44	(66)			
Difco .....	12	— 95	— 50	— 168	— 33	— 200	— 17	— 40	—			
Milliliters of acid simultaneously produced												
Nutrimin .....	0.4	(0.4) 0.8	(0.8) 3.6	(6.4) 7.6	(16.3) 0.6	(1.0) 1.1	(2.4) 0.6	(0.6) 0.8	(0.8)			
Aminosol .....	1.4	— 1.8	— 8.0	— 17.2	— 1.1	— 1.9	— 1.4	— 1.6	—			
Astra A .....	0.9	(0.9) 1.2	(1.3) 3.1	(12.0) 8.4	(21.7) 1.1	(1.8) 2.2	(8.5) 0.9	(1.4) 1.2	(1.7)			
Astra C .....	0.8	(1.0) 0.8	(0.8) 0.9	(1.4) 1.5	(3.2) 0.9	(1.0) 1.0	(1.1) 0.9	(1.0) 0.8	(1.0)			
Wilson L .....	1.4	(1.5) 1.8	(1.8) 1.6	(15.8) 6.6	(21.8) 1.6	(2.1) 3.3	(15.8) 1.6	(2.1) 2.2	(2.8)			
Organon A .....	0.8	(1.0) 0.8	(0.9) 3.7	(5.3) 7.0	(7.3) 1.8	(2.4) 5.2	(4.1) 0.8	(1.1) 1.0	(1.4)			
Organon B .....	0.9	(0.7) 1.0	(0.8) 14.2	(14.9) 21.8	(23.3) 1.8	(2.7) 7.5	(6.6) 1.5	(1.9) 1.4	(1.9)			
Difco .....	1.0	— 2.0	— 2.4	— 9.2	— 1.5	— 11.4	— 1.4	— 2.0	—			

Table 11.

*Growth of Lactobacillus lactis 100 after adding extracts containing the anti pernicious-anemia liver factor and (or) streptogenin to the basal medium of HENRIKSEN and SELL. Medium autoclaved 5 minutes at 15 pounds pressure. Extracts autoclaved 30 minutes at the same pressure or added aseptically.*

Preparations	Photoelectric readings after 24 and 48 hours of incubation. <sup>1</sup> The amounts of substances added per tube were the following									
	0		4.8 mg.		0.18 mg.		0.0018 mg.			
	24	48	24	48	24	48	24	48	24	48
Aminosol .....	4	4	27	55	14	19	12	20	(25)	
Astra A .....	(18)	(34)	(30)	(160)	(53)	(48)	(112)	(20)	(41)	(39)
Milliliters of acid simultaneously produced <sup>2</sup>										
Aminosol .....	0.4	(1.2)	1.6	3.2	1.2	(1.2)	1.2	(1.1)	1.3	(1.3)
Astra A .....	1.1	(0.8)	10.8	(8.8)	(16.4)	(1.7)	(5.8)	(1.0)	1.7	(1.9)

<sup>1</sup> Given as scale readings on the Klett-Summerson photoelectric colorimeter. The values in parenthesis are those obtained after aseptical addition of filter-sterilized solutions. Mean values from three tubes corrected for blank readings.

<sup>2</sup> Given as ml. of 0.05 N NaOH to titrate 6 ml. of final solution. The values are the mean values from three tubes corrected for the blank titrations.

pogenin and other less characterized factors and what should be ascribed to the anti-pernicious-anemia factor present in the material. To test the specificity of *Lactobacillus lactis* in this respect the microorganism was cultivated in the medium supplemented with the fractions from electrolyzed Heptomin. Results of a typical experiment are shown in Table 6b. Comparing the 24 hours figures of this experiment with those of the experiments carried out on *Lactobacillus citrovorum* and *Lactobacillus casei* (Tables 6 and 6a) some differences are observed. All the three fractions had about the same growth stimulating activity when tested on *Lactobacillus citrovorum* and *Lactobacillus lactis* while the anode fraction had the definite stronger growth effect when tried on *Lactobacillus casei*. The growth figures obtained when the electrolyzed fractions were tested on *Lactobacillus lactis* were usually higher than those obtained with the two other microorganisms.

*Discussion.* A thorough interpretation of the results reported in this paper must await the identification of the several growth factors obviously present in the investigated extracts. One of the main topics of the investigation was to test to some extent the reliability of using certain lactobacilli for amino acid analysis in extracts of animal origin. Growth factors of unknown composition present in such material could complicate the quantitative analysis. Liver was chosen as test material since most of the microbial growth factors so far identified and also many less characterized have been shown to be present in liver extracts. The growth of microorganisms as *Lactobacillus arabinosus*, *Streptococcus faecalis* R (8043), *Streptococcus lactis* (9790) and *Leuconostoc mesenteroides* were not considerably stimulated by the addition of liver extracts to the synthetic growth medium used in the present investigation, especially when incubation times of 48 and 72 hours was used. However, this conclusion may not be directly transferable when other synthetic media are employed. Experiments carried out in this laboratory with synthetic peptides added to different synthetic media show that the peptides may be differently utilized.<sup>1</sup>

Another topic of the present investigation was to estimate the amounts of growth factors present in liver extracts and enzyme hydrolysates or proteins which stimulated certain lactobacilli, *Lactobacillus citrovorum* (8081), *Lactobacillus casei*, *Lactobacillus acidophilus* and *Lactobacillus lactis* 100. As a source of streptogenin, which *a priori* could be suspected to be present, enzymatic hydro-

<sup>1</sup> ÅGREN, G., unpublished data.



lysates of commercial casein were used. They were also active for the four last mentioned microorganisms. However, strepogenin may not be the only growth factor present in such enzymatic digests. It is a well known fact that CASTLE and coworkers have demonstrated that extrinsic factor is present in commercial casein preparations, but if this factor is left intact in the tryptic digestion of casein is so far unknown. The possible rôle of extrinsic factor as microbial growth factor has not been investigated. As a glutamic acid containing peptide it was thought that strepogenin would move to the anode compartment of the electrodialysing apparatus used in the present investigation. It could also be demonstrated in experiments with *Lactobacillus casei* that the acid solution of this compartment contained a growth factor for this microorganism. However, the middle and cathode compartments also contained growth stimulating factors. Electrodialysis was also used to separate strepogenin if present in different types of liver preparations. A refined preparation, Heptomin, used in the treatment of pernicious anemia, was in this way separated in three fractions, an acid of pH 3.5, a more neutral (middle) fraction of pH 5.5 and a cathode fraction of pH 7.9. Each of the three fractions contained factors which stimulated the growth of *Lactobacillus citrovorum*, *Lactobacillus casei*, *Lactobacillus acidophilus* and *Lactobacillus lactis*. Preliminary experiments carried out in this laboratory<sup>1</sup> seemed to demonstrate a connection between the growth factor present in the anode compartment after electrodialysing an enzymatic casein hydrolysate and that obtained in the same way from Heptomin. Other experiments performed with a modification of the biuret reaction applied to paper partition chromatography in this laboratory<sup>2</sup> showed that only the cathode fraction of Heptomin contained a substance giving a biuret positive spot, besides of several ninhydrine positive. Since the anti-pernicious-anemia factor chiefly remained in the middle fraction after the electrodialysis this seems to show that this factor has not a polypeptide structure. The anode fraction from electrodialysis of casein hydrolysates or Heptomin showed after the development of a partition chromatography only ninhydrine positive spots. The negative biuret reaction of this fraction is of interest with regard to the present discussion of the structure of strepogenin (WOOLLEY, 1948). The question of whether the activity of each fraction from

<sup>1</sup> ÅGREN, G., and DE VERDIER, K., unpublished data.

<sup>2</sup> KILLANDER, A., unpublished data.

the electrodialysis of the liver extract (Heptomin) is represented by a single factor is not known. This problem is under investigation in this laboratory. So far it has been shown that the three fractions obtained by electrodialysing the Wilson fraction L all contained growth factors for *Lactobacillus casei*. This preparation is supposed to be free from or only containing traces of the anti-pernicious-anemia factor. Accordingly, the presence of at least four unidentified growth factors in liver extracts seems probable. The relationship of any of these factors to the animal protein factor of SCOTT and al. is not known.

### Summary.

Studies have been conducted on the possible growth factor requirements of twelve lactobacilli, several of them used in the microbiological amino acid analysis. Liver extracts and enzyme hydrolysates of casein were used as sources of growth factors. It was shown that *Lactobacillus citrovorum* (8081), *Lactobacillus casei*, *Lactobacillus acidophilus* and *Lactobacillus lactis* 100 required a streptogenin similar factor present in both types of materials. Besides, liver extracts containing the anti-pernicious-anemia factor were separated in three fractions all of which contained growth factors for these four microorganisms. The possible bearing of the results on the microbiological analysis of amino acids is emphasized. The possibility of using any of the four mentioned microorganisms for the standardization of the anti-pernicious-anemia factor is analyzed and discussed. Some of the properties of this factor are also discussed in the light of the results obtained.

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From the Department of Physiology, University of Lund.

# **The Occurrence of Citric Acid in the Shell Substance of Eggs of Chickens, Ducks, and Geese.**

By

**T. THUNBERG,**  
Lund.

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The presence of Ci (= citric acid or citrate) in significant amounts in the shells of birds' eggs was demonstrated in 1941 by the author. This report was based on the analyses of egg shells of about ten different avian species with a Ci-content of 0.8—3.5 pro mille or 800—3,500 p. p. m. (= parts per million). This latter method of representation which eliminates the use of decimals when expressing such small concentrations as the above is already widely employed in the U. S. A.

It must be pointed out at once, however, that no great significance can be attributed to the observed differences in the Ci-content of egg shells from different species of birds, partly because it was impossible to determine definitely which stage of the development of the egg the investigated shell represented. In most cases the eggs examined were taken from museum collections. The conclusion could be drawn without hesitation, however, that Ci is a normal constituent of the shell of bird's eggs and that it occurs there in a concentration of around 2 pro mille. The investigation also provided the answer to the question as to whether biological deposits containing calcium phosphate also regularly contain Ci. The answer is obviously in the affirmative.

In addition to the above-mentioned values there are a few solitary ones taken partly from THUNBERG (1941) and partly from DICKENS. The latter are of special interest as they elucidate the question of whether the Ci-concentration in the newly-laid egg is sufficient to supply the Ci-content of the body of the newly-

hatched chick, and this seems to be the case. These data, of course, do not prove that the Ci-storage of the chick is directly received from the shell. Ci may be synthetized during the course of the chick's development.

The experiments reported below have been carried out according to the method of PUCHER, SHERMAN and VICKERY as modified by HUNTER and LELOIR and it is described in detail by the author in an earlier paper (1948).

The material for the analyses was composed of 30 chicken eggs, 6 duck eggs, and 6 goose eggs. The eggs were fresh, which could easily be determined when they were freed from the shell. At least some of the eggs were taken from the nest immediately after they were laid. Before analysis the egg shell was pulverized, dried, and extracted with petroleum ether.

The results of the experiments are shown in the following table. The Ci-values of the individual analyses are not given, only the average figures and the range expressed in p. p. m.

Table I.

Egg shell	Average	Range
Chicken .....	1,496	500
Duck .....	411	39
Goose .....	309	17

Among the 30 analyses of the shell of chicken eggs there was a group of 4 which gave considerably higher values than the others, thus greatly increasing the average value. If these figures are omitted the average value becomes 1,315 p. p. m.

Whether one employs the higher or lower average the Ci-content of the shell of chicken eggs remains considerably above the values for duck and goose eggs, which were 411 and 309 respectively. The Ci-content of goose eggs is thus the lowest. The range values show that these figures are significant.

The absolute size of the eggs examined was not recorded. When such values were desired they were taken from the literature. An average value of sufficient accuracy was obtained for the chicken egg of 55 g. The average values of 60 g. for duck eggs and 150 g. for goose eggs are noted only with hesitation (cf. KÖNIG). If the lowest values of the properties in question, the Ci-content and the weight, are represented as equal to 1 the following results are obtained:

<i>Citrate series:</i>	1	1.25	5
<i>Weight series:</i>	2.7	1.70	1

A glance at these values immediately raises the question of whether this is a case of negative correlation. The situation deserves, however, a more detailed examination.

The chemistry of the egg shell may seem to be of little significance but when working with this problem one soon finds that there are many questions of theoretical as well as practical interest. The factors which influence the hardness of the egg shell also effect the ability of the egg to stand transportation. The distance between the site of production and the place of consumption is often long and the risk for mechanical damage considerable. Then there is the question of the most suitable feed. Does the chicken require more citric acid in its feed?

### Summary.

The author has investigated the percentual Ci-content of shells from chicken, duck, and goose eggs. The citric acid analyses have been carried out according to the method of HUNTER and LELOIR. The experimental material was composed of 30 chicken eggs, 6 duck eggs, and 6 goose eggs. The average Ci-content in somewhat rounded figures of chicken eggs was found to be 1.5 mg. per g., of duck eggs 0.4 mg., and of goose eggs 0.3 mg., or expressed in p. p. m., 1,500, 400, and 300. A negative correlation seems to exist between the weight of the egg and the Ci-content of the egg shell. The author stresses the practical importance of knowledge of the factors influencing the Ci-content of the shell.

The author is indebted to Amanuens Johannesen for his assistance with the statistical calculations and to Miss Birgit Lundqvist for her help with the analyses.

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From the Department of Physiology, University of Lund.

## **The Limits of Error of the Assay of Pregnant Mares' Serum Gonadotropin by the Ovarian Weight Method.**

By

DORA JACOBSON.

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Since the pioneer work of TREVAN, BURN and GADDUM a number of statistical methods applicable to biological assays have been presented (IRWIN, 1937, BLISS and MARKS, 1939, IPSEN, 1941). The need for a statistical analysis of the result of a biological assay is obvious. The application of statistical methods to the assay of gonadotropic hormones has not however been very extensive.

In a recent paper on the assay of pregnant mares' serum gonadotropin using the chick as test animal DORFMAN et al., 1948, point out, that whereas a number of methods are being employed for the assay of equine gonadotropin "no systematic statistical analysis for errors of determination have been presented".

A direct and objectively measurable response suitable for the assay of pregnant mares' serum gonadotropin is given by the ovarian weight of immature rats (CARTLAND and NELSON, 1938, HAMBURGER and PEDERSEN-BJERGAARD, 1937). The ovarian weight test is widely employed and relatively simple when using the technique of the present investigation. Therefore an account of the limits of error of assays performed by this method is presented here. Special attention is devoted to the question of the design of the assay and to the question as to whether the standard preparation and the unknown have to be tested simultaneously.

*Experimental. Material:* The Material consists of 9 different preparations. The results of 16 assays performed during the period from October 1946 until May 1948<sup>1</sup> are analysed statistically. In 5 cases the potency of the unknown preparation is determined by computing the values obtained from 2 or 3 assays resp.

The preparations used are 1) 7 commercial concentrates, Gestyl, Pharmacia, 2) 1 commercial concentrate, Antex, Løvens<sup>2</sup> and 3) the international standard preparation made from pregnant mares' serum. All preparations are completely soluble in water.

*Methods:* The assay is performed on inbred immature female albino rats fed on a uniform diet. At the beginning of the assay the animals are 23—28 days old; their body weight is about 35 gm. — A solution of the material in a suitable dilution with physiological saline is prepared immediately before use. With the exception of 1 assay (preparation G 15) in which 5 doses each of 0.2 cc are given within 48 hours, each animal is injected with a single dose of 1.0 cc subcutaneously (COLE and ERWAY, 1941, ALBERT and MONEY, 1946). — 96 hours (CARTLAND and NELSON, 1938) after the first injection both ovaries are removed, and weighed immediately on a torsion balance (1 interval = 1 mg).

The design and the calculation is made according to BLISS and MARKS (1939). Where the requirements for a factorial analysis are not fulfilled, the results are analysed according to IRWIN (1937) or IPSSEN (1941). The standard and the unknown preparation are tested simultaneously, the number (2<sup>3</sup> or 3) of doses and the interval between doses (according to a logarithmic scale) being equal for the standard and the unknown preparation. As far as possible the 4 or 6 doses employed are distributed equally among litter mates. Care is taken to apply doses giving responses within the straight part of the log. dose-response curve; that is in my material for the mean weight of both ovaries covering a range from about 25 to 95 mg. In all cases the log. dose-response curves of the standard and unknown tested simultaneously are parallel.

The calculations employed for this investigation are explained in the papers mentioned above, and in a recent publication by PUGSLEY (1946). Therefore a numeric example only will be given for each of the two methods adopted (tables 1 and 2).

*Result.* The results are summarized in tables 3 to 5. Table 3 shows the relation between the number of animals and the limits of error of the assay (col. 4 and 5, and 7). The limits of error are determined for  $P = 0.05$  (see IRWIN, 1943).

<sup>1</sup> I am indebted to dr. med. FREDERIK PAULSEN for permission to include material obtained from standardizations I made while working for the Nordiska Organon, Stockholm.

<sup>2</sup> Exp. with this material were performed, concomitantly with other laboratories with the aim of arriving at a Scandinavian standard preparation for the gonadotropic hormone from pregnant mares' serum.

<sup>3</sup> 2 doses each of standard and unknown are employed, when both are giving responses within the straight part of the log. dose-response curve, and when the mean ovarian weight corresponding to the two doses is on either side of 50 mg.

Table 1. Example according to Bliss and Marks. Preparat. A. H. (U) and Int. stand. serumgonad. (S).

Dose mg	Log dose	Number of responses	S(y)	S(y <sup>2</sup> )	S <sup>2&gt;y</sup> n
S <sub>1</sub>	2.0	8	215	5 939	5 778.125
S <sub>2</sub>	4.0	8	469	28 926	27 495.125
S <sub>3</sub>	8.0	8	854	92 676	91 164.500
U <sub>1</sub>	0.87506	—1	206	5 574	5 304.500
U <sub>2</sub>	0.17609	8	493	37 039	30 381.120
U <sub>3</sub>	0.47712	8	879	100 089	96 580.130
		48	3 116	270 242	256 703.500
			—202 280	—202 280.000	—202 280.000
			67 962		54 423.500

$$\frac{S^2y}{n} = \frac{9\ 709\ 456}{48} = 202\ 280$$

### Analysis of variance

Degrees of freedom	Sum of squares	Mean square of variance
5	54 423.5	10 884.70
42	13 538.5	322.35 = s <sup>2</sup>
47	67 962.0	s = 17.954

Variance due to:  
Differences between doses  
Differences within doses  
Total

### Factorial analysis

Factorial coefficients (x) for dose	U <sub>1</sub>	U <sub>2</sub>	U <sub>3</sub>	NS(x <sup>2</sup> )	S(xYp)	S <sup>2</sup> (xYp) NS(x <sup>2</sup> )	Standard Dev.	Variance ratio F
S <sub>1</sub>	—1	—1	—1	48	—40	33.33 = D <sup>2</sup>	5.77 = D	0.103
S <sub>2</sub>	—1	—1	—1	32	—34	53 792.90 = B <sup>2</sup>	231.93 = B	166.875
S <sub>3</sub>	—1	—1	—1	32	—34	36.13		0.112
Slope of dose effect curve	—1	—1	—1	96	—230	551.04		1.709
Departure from parallelism	—1	—2	—1	96	—32	10.67		
Curvature .....	—1	—2	—1	96	—32			
Opposed curvature .....	—1	—2	—1	96	—32			
Total of responses on each dose:	215	469	854	206	493	879		

$$\frac{S(xYp)}{n} = \frac{1\ 312}{215} = 6.097$$

$$b = \frac{S(xYp)}{NS(x^2)} = \frac{0.30103 \cdot 32}{136.199} = 0.0122; \text{antilog} = 1.020.$$

$$M = \frac{KID}{B} = \frac{1.633 \cdot 0.30103 \cdot 5.77}{231.93} = 0.0122; \text{antilog} = 1.020.$$

$$\text{With the standard at 100 \% the sample} = 103 \%$$

$$\text{Limits of error } s_M = \frac{B^2}{sKI/VB^2 + D^2} = \frac{53\ 792}{17.954 \cdot 1.633 \cdot 0.30103 \sqrt{33.33 + 53\ 792}} = 0.038065.$$

$$\text{Log } s_M \text{ at } P = 0.05 = \pm 0.038 \cdot 2.031 = 0.0768 \text{ antilog} = 1.193$$

$$\text{Log } s_M \text{ at } P = 0.05 = \pm 0.038 \cdot 2.031 = 0.0768 \text{ antilog} = 0.838.$$

$$\text{Limits of error at } t = 0.05 \text{ are } 83.8 \% \text{ to } 119.3 \%$$

The experiments are divided into 4 series: 1) nrs. 1—4 with the limits of error of more than  $\pm 25\%$  of the calculated potency (col. 6), 2) nrs. 5—7 with those of between  $\pm 25\%$  and  $\pm 20\%$ , 3) nrs. 8—14 with those of between  $\pm 20\%$  and  $\pm 15\%$ , and 4) nrs. 15—20 with the limits of error of  $\pm 15\%$  or less.

Series 1 and 2 show 5 assays performed with 3 doses and 2 with 2 doses each of the standard and the unknown preparation. With the exception of nr. 4 with 57 animals a number of 30 to 40 test animals are employed for the assay.

Series 3 includes the results of 5 assays with 3 doses and of 2 with 2 doses. Except experiment 8 and 14, performed on 27 and 72 animals resp., the number of test animals is between 38 and 63.

Series 4 summarizes 4 assays with 3 doses and 2 with 2 doses. The average number of test animals is 76. However the experiments 15, 16 and 17 show that the level of precision of  $\pm 15\%$  may be obtained with 48 to 60 responses.

The results summarized in table 3 demonstrate the well known fact that the level of precision increases with the number of responses. The exceptions (exp. 4, 8, 14, 15, 16, 17) show that this does not always hold true, however.

Table 4 shows the variations of the standard deviation of a single observation (= square root of intra group variance,  $s$ ; see col. 5) and of the slope of the dose-response curve (= regression coefficient,  $b$ ; see col. 6) for 16 assays of 7 different preparations performed at different periods from October 1946 until May 1948.

The range for the values of the intra-group variance,  $s$ , is  $10 \pm 0.5$  to  $18 \pm 2$ . The range for the values of the slope of the log. dose-response curve,  $b$ , is  $70 \pm 8$  to  $136 \pm 11$ . Obviously there is a significant difference between these values. — The regression coefficient of exp. 1 is not considered, as the animals had been treated with divided doses.

An investigation of the significance of the differences between the slopes of log. dose-response curves of assays performed at different times on one and the same preparation is summarized in table 5. As the principles and calculations for the comparison of reaction lines with FISHER's  $z$  test are explained by *e. g.* IPSSEN (1941) they will not be repeated here. The calculations are made on the results obtained from assays within the experiment nrs. 4—7 quoted in table 4. An example for the calculation of the differences between the regression coefficients of exp. 7 b, 7 c, 7 d is given in table 6.



Table  
Example according

			Int. stand.						
Dose mg	Log dose	Working units	n	$\Sigma x$	$\Sigma x^2$	$\Sigma y$	$\Sigma y^2$	$\Sigma xy$	$\frac{1}{n} \cdot (\Sigma y)^2$
2.0	0.30103	-1	4	-4	4	99	2 489	-99	2 450.25
4.0	0.60206	0	4	0	0	257	16 669	0	16 512.25
8.0	0.90309	1	4	4	4	382	37 570	382	36 481.00
			12	0	8	738	56 728	283	55 443.50
							<u>-45 387</u>		<u>-45 387.00</u>
						SS <sub>y</sub> = 11 341			10 056.50

$$SS_x = 8$$

$$\bar{y} = 61.5$$

$$\frac{1}{n} \cdot (\Sigma y)^2 = 45\,387$$

$$SP_{xy} = 283 \quad b = \frac{283}{8} = 35.4 \quad \text{in real units } b = 117.6 \pm 13.3.$$

$$SS_{y \cdot x} = 11\,341 - 35.4 \cdot 283 = 11\,341 - 10\,018.2 = 1\,322.8.$$

$$MS_{y \cdot x} = 132.28 \quad s_b = \sqrt{16.54} = \pm 4 \quad \text{in real units } s_b = 13.3.$$

#### Analysis of variance

Variance due to:	Degrees of freedom	Sum of squares
Differences between doses .....	2	10 056.50
Differences within doses .....	9	1 284.50
Total	11	11 341.00

$$s^2 = \frac{1284.5 + 812.4}{9 + 12} = 99.85$$

$$t = \frac{5.3}{\sqrt{169 + 64}} = 0.353 \text{ no sig.}$$

$$\bar{b} = \frac{621}{18} = 34.5 \quad \text{in real units } \bar{b} = 114.6 \pm 7.4.$$

$$s_b^2 = \frac{1}{24} \cdot \frac{23\,589 - 34.5 \cdot 621}{18} = 5.01 \quad s_b = 2.24 \quad \text{in real units } s_b = 7.44$$

$$M = \frac{8.1}{34.5} = 0.2348 \quad \text{in real units } M = 0.07068 \quad \text{antilog} = 1.176.$$

With the standard at 100 % the sample is 118 %

$$s_M^2 = \frac{1}{1\,190.25} \cdot \left[ 11.02 + 4.22 + \frac{65.61}{1\,190.25} \cdot 5.01 \right] = 0.01304;$$

$$s_M \text{ in real units at } P = 0.05 = \pm 0.2371 \cdot 0.30103 = 0.07137 = 0.92863 - 1$$

Table 5 shows that the slope of the log. dose-response curves of the three assays included in exp. 4 and 5 resp. of table 4 can be regarded as identical (probability more than 5 per cent). The interval between these assays is 18 and 16 days resp.

For exp. 6 a, 6 b and 6 c the probability for the 3 slopes to be

2.

to Ipsen

G. Ph.

Dose mg	Log dose	Working units	n	$\Sigma x$	$\Sigma x^2$	$\Sigma y$	$\Sigma y^2$	$\Sigma xy$	$\frac{1}{n} \cdot (\Sigma y)^2$
1.2	0.07918	-1	5	-5	5	101	2 109	-101	2 040.2
2.4	0.38021	0	5	0	0	261	13 837	0	13 624.2
4.8	0.68124	1	5	5	5	439	39 075	439	38 544.2
			15	0	10	801	55 021.0	338	54 208.6
							<u>-42 773.4</u>		<u>-42 773.4</u>
							$SS_y = 12\,247.6$		11 435.2

$$SS_x = 10$$

$$\bar{y} = 53.4$$

$$\frac{1}{n} \cdot (\Sigma y)^2 = 42\,773.4$$

$$SP_{xy} = 338 \quad b = 33.8 \quad \text{in real units } b = 112.3 \pm 8.3$$

$$SS_{y/x} = 12\,247.6 - 33.8 \cdot 338 = 823.2.$$

$$MS_{y/x} = 63.3 \quad s_b = \sqrt{6.33} = \pm 2.5 \quad \text{in real units } s_b = 8.3.$$

# Analysis of variance

Variance due to:	Degrees of freedom	Sum of squares
Differences between doses .....	2	11 435.2
Differences within doses .....	12	812.4
Total	14	12 247.6

$$s = 10 \quad V_s = \frac{99.85}{42} = 2.377.$$

nificant difference between the two slopes

$$s_M = \pm 0.114; \text{ at } P = 0.05 \quad s_M = \pm 0.114 \cdot 2.08 = \pm 0.2371$$

antilog = 1.178. At  $P = 0.05$  the limits of error are 85 % to 118 %.

$$s = 0.848$$

identical is less than 5 % but more than 1 %. The interval between the assays is 56 days.

When one of the values is discarded we find a significant difference between the slopes of the reaction lines of exp. 6 b and 6 c (interval for the assays 38 days), whereas those of 6 a

**Table 3.**  
*Showing number of animals and limits of error.*

Exp. nr.	Preparation		Number of doses for standard and unknown	Number of animals for		Potency in % of standard	Limits of error of the assay (P = 0.05)
	Standard	Unknown		standard	unknown		
1	G. Ph.	G. 20	3	15	15	100	± 25 % or more
2	Int. stand.	A. H.	3	18	15	117	77—130
3	G. 39	G. 18	2	20	20	121	77—130
4	Int. stand.	G. 17 II	3	28	29	105	78—128
							76—131
5	G. 39	G. 18	2	16	16	106	± 20 to ± 25 %
6	Int. stand.	G. Ph.	3	18	15	125	79—126
7	Int. stand.	G. Ph.	3	18	18	90	81—123
							81—123
8	Int. stand.	G. Ph.	3	12	15	118	± 15 to ± 20 %
9	G. 39	G. 18	2	19	19	67	85—118
10	Int. stand.	A. H.	3	24	24	103	83—121
11	Int. stand.	A. H.	3	24	24	144	84—119
12	G. 39	G. 17 I	3	27	26	121	84—119
13 (a)	Int. stand.	G. Ph.	3	30	33	112	83—121
14 (b)	G. 39	G. 18	2	36	36	107	85—117
							86—117
15	Int. stand.	A. H.	3	24	24	115	± 15 % or less
16	G. 39	G. 15	2	28	28	86	86—116
17	G. Ph.	G. 20	3	30	30	108	88—113
18 (c)	G. Ph.	G. 20	3	45	45	105	87—114
19 (d)	Int. stand.	A. H.	3	48	48	129	88—114
20 (e)	G. 39	G. 18	2	55	55	81	89—112
							88—114

See table 4: a) = exp. 6 a + b

b) = " 4 a + c

c) = " 5 b + c

d) = " 7 c + d

e) = " 4 a + b + c

and 6 b with a time interval of 18 days can be regarded as identical.

The difference between the 4 regression coefficients of exp. 7 a—7 d, extending over 57 days, is significant. Even for exp. 7 b—7 d with an interval of 44 days the probability of the 3 slopes to be identical is less than 1 %. The reaction lines are not parallel.

The log. dose-response curves of exp. 7 c and 7 d, performed within 10 days, are parallel, however.

The results show that the reaction curves of assays made on one and the same preparation at different times are not always parallel. In the present material a significant difference is found, when the interval between the assays exceeds 4 weeks.

Table 4.

Showing intra-group variance and regression coefficient within assays performed at different times.

Nr.	Standard	Unknown	Number of test animals	Square root of intra-group variance, s	Regression coefficient, b	Assay performed
1 <sup>1</sup> (16)	G. 39	G. 15	56	13 ± 0.4	139 ± 8	<sup>10</sup> / <sub>10</sub> 1946
2 (12)	Int. stand.	G. 17 I	53	13 ± 0.5	91 ± 8	<sup>16</sup> / <sub>4</sub> 1947
3 (4)	Int. stand.	G. 17 II	57	17 ± 0.5	76 ± 10	<sup>9</sup> / <sub>3</sub> »
4 a (5)	G. 39	G. 18	32	12 ± 0.5	87 ± 10	<sup>20</sup> / <sub>10</sub> »
b (9)	G. 39	G. 18	38	10 ± 0.4	90 ± 11	<sup>1</sup> / <sub>11</sub> »
c (3)	G. 39	G. 18	40	11 ± 0.4	70 ± 8	<sup>7</sup> / <sub>11</sub> »
5 a	G. Ph.	G. 20	30	15 ± 0.7	84 ± 11	<sup>24</sup> / <sub>2</sub> 1948
b (17)	G. Ph.	G. 20	60	13 ± 0.4	109 ± 7	<sup>3</sup> / <sub>3</sub> »
c (1)	G. Ph.	G. 20	30	14 ± 0.7	94 ± 11	<sup>11</sup> / <sub>3</sub> »
6 a (7)	Int. stand.	G. Ph.	36	14 ± 2	103 ± 9	<sup>22</sup> / <sub>1</sub> »
b (8)	Int. stand.	G. Ph.	27	10 ± 0.5	115 ± 7	<sup>9</sup> / <sub>2</sub> »
c (6)	Int. stand.	G. Ph.	33	10 ± 0.4	79 ± 7	<sup>18</sup> / <sub>3</sub> »
7 a (2)	Int. stand.	A. H.	33	11 ± 1.5	76 ± 9	<sup>18</sup> / <sub>3</sub> »
b (10)	Int. stand.	A. H.	48	18 ± 2	136 ± 11	<sup>1</sup> / <sub>4</sub> »
c (11)	Int. stand.	A. H.	48	13 ± 1.4	103 ± 7	<sup>4</sup> / <sub>5</sub> »
d (15)	Int. stand.	A. H.	48	11 ± 0.4	100 ± 7	<sup>14</sup> / <sub>5</sub> »

<sup>1</sup> The preparation is given in 5 doses of 0.2 cc.

( ) = exp. number of table 3.

*Discussion and Conclusions.* The statistical analysis of the results obtained from the assay of the gonadotropic activity of preparations made from pregnant mares' serum shows that the ovarian weight method allows the potency of an unknown preparation to be estimated at P 0.05 with a precision of ± 15 % or less, when a number of about 30 to 45 animals are used for the standard and for the unknown preparation.

If the standard deviation of a single observation (= square root of intra group variance, s) and the slope of the log. dose-response curve (= regression coefficient, b) are known, the precision of the result of the assay (= standard error of the log. ratios of potencies,  $s_M$ , = limits of error) can be predicted according to

the formula  $s_M = \frac{s}{b} \cdot \sqrt{\frac{2}{N'}}$ , where  $N'$  is the number of responses for each of the two preparations (see BLISS and MARKS, 1939).

The results obtained in the present investigation on equine gonadotropin show, that the values for s as well as for b undergo variations. The claim, as put forward by e. g. BLISS and CATTELL, 1943, of testing the unknown preparation "together with the

Table 5.

Showing comparison of slopes within assays performed on 4 different preparations at different times (see table 4 experiments 4 to 7).  
*z* test (Ipsen loc. cit. p. 38—41).

Comparison of experiments: (see table 4)	$\Sigma n$	$\Sigma n_b$	$MS_b$	$MS_{y/x}$	$z$	Probability of the slopes to be identical	
4 a, 4 b, 4 c...	110	3	229	115	0.336	> 0.05	
5 a, 5 b, 5 c...	120	3	404	185	0.392	> 0.05	
6 a, 6 b, 6 c...	96	3	625	141	0.742	> 0.01	< 0.05
6 a, 6 b .....	63	2	126	165	0.131	> 0.05	
6 b, 6 c .....	60	2	1 159	92	1.264		< 0.01
6 a, 6 c .....	69	2	614	162	0.668	> 0.01	< 0.05
7 a, 7 b, 7 c, 7 d	177	4	1 655	208	1.037		< 0.01
7 b, 7 c, 7 d...	144	3	1 147	224	0.815		< 0.01
7 c, 7 d .....	96	2	10	177	1.435	> 0.05	
7 a, 7 c, 7 d...	129	3	674	170	0.688	> 0.01	< 0.05

$n$  = number of points for 1 curve.

$n_b$  = number of curves.

$MS_b$  = mean square of the deviations of  $n_b$  slopes from the mean slope.

$MS_{y/x}$  = mean deviation of all points from the reaction lines.

$z$  = natural logarithm of the square root of the quotient from  $MS_b$  and  $MS_{y/x}$  (the greatest value is divided by the smallest).

The probability of the slopes to be identical is estimated from the tables of Fisher and Yates: Distribution of  $z$  5 Per Cent Points and 1 Per Cent Points (see Fisher and Yates loc. cit. p. 38 and 40).

standard under as nearly identical conditions as possible" should therefore be respected when working with gonadotropic hormones. Because of the variations in the susceptibility of the rat's ovary to the gonadotropic hormone (s and b), a result based on a comparison of the potencies of a standard and an unknown preparation, where the standard is not tested simultaneously, may give rise to misleading conclusions, both with respect to the potency of the unknown preparation and to the precision of the assay.

With respect to the design of the assays the following procedure might be recommended:

1) By means of a preliminary test on a small number of rats, doses giving a mean ovarian weight within the straight part of the log. dose-response curve are determined both for the standard and for the unknown preparation.

2) Three (or two) according to a logarithmic scale increasing doses each of standard and unknown are tested on a number of animals known by previous experience to be sufficient for ob-

Table 6.

Example for the calculation of the dates presented in table 5.  
(See Ipsen, 1941).

Exp.	n	$\frac{SP_{xy}^2}{SS_x}$	$SS_y$	$SS_{y/x}$
7 b	48	$\frac{1312^2}{32} = 53\ 792$	67 962	14 170
7 c	48	$\frac{994^2}{32} = 30\ 876$	41 355	10 479
7 d	48	$\frac{968^2}{32} = 29\ 282$	<u>35 460</u>	<u>6 198</u>
			$\Sigma SS_y = 144\ 797$	$\Sigma SS_{y/x} = 30\ 847$
			$\frac{(\Sigma P_{xy})^2}{\Sigma SS_x} = \frac{3\ 274^2}{96} = 111\ 657$	
			$MS_{y/x} = \frac{30\ 847}{138} = 223.53$	
			$MS_b = \frac{1}{2} (144\ 797 - 30\ 847 - 111\ 657) = 1\ 146.5$	
			$z = \log_n \sqrt{\frac{1\ 146.5}{223.53}} = \log_n 2.26 = 0.815.$	

In the table of Fisher and Yates the value for  $z$  corresponding to  $n_1 = 2$  and  $n_2 = 138$  is 0.5486 for the probability of 5 per cent, and 0.7636 for the probability of 1 per cent. As the value of  $z$  found in the present experiment is larger than 0.7636 the probability for the slopes to be identical is less than 1 per cent.

taining the level of precision required. The responses should be as nearly identical as possible for the corresponding doses of the standard and unknown.

3) The result obtained from 2) is analysed statistically. If the precision of the assay is not satisfactory,

4) the number of responses required for the specified level of precision is estimated according to the equation given above,

which is rearranged  $N' = \frac{2}{s_M^2} \cdot \frac{s^2}{b^2}$ , and the assay continued accordingly. The time interval between assays to be computed should be as short as possible.

The level of precision required should be determined before the assay is begun, and the design made according to the precision required.

### Summary.

1) The precision of the assay of pregnant mares' serum gonadotropin with the ovarian weight method is determined. With

the material and method employed for the investigation presented, the limits of error of the assay are  $\pm 15\%$  or less ( $P = 0.05$ ), when a number of 30 to 45 animals are used each for the standard and the unknown preparation.

2) The susceptibility of the rats' ovary is variable. Therefore the potency of a preparation should be determined by simultaneously testing the standard and the unknown.

3) A design for the assay is described. The design should be made with due regard to the level of precision required.

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## Studies on the Absorption of Iron.<sup>1</sup>

### II. Experimental Studies on the Serum Iron Level in the Porta Vein during Iron Absorption Tests.

By

GÖSTA NEANDER and BO VAHLQUIST.

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Absorption tests are nowadays used widely when investigating the absorption and the utilization of different substances, such as sugar, protein, fat, vitamins and minerals.

The absorption curve is based on the results of analyses of the blood in the peripheric vessels. It is, however, *a priori* to assume that the concentration of those substances which are more or less completely absorbed by the porta blood system, might be altered during the passage through the liver. This organ has a central position also in the metabolism of iron, being not only a storage depôt but probably also exerting a regulating influence on the metabolism of the metal. It seemed to us to be an interesting problem to compare the behavior of serum iron in porta blood with that of the blood in other vessels during iron absorption.

*Material and technique:* White rabbits weighing 2—2.5 kg were starved for 24 hours. Blood samples were collected from the vessels of the ear. 100 mg of ferrous iron in the form of pyrophosphate ("Gut-tafer") were administered with stomach tube. After varying intervals, usually 20—60 minutes, laparotomy was performed, the animals previously anesthetized with "Numal" Roche 0.5 cc pro kg body weight intravenously.

Blood samples were taken immediately after laparotomy from vena

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<sup>1</sup> Part I of this series: "Absorption of iron from the stomach" is published in *Acta paediatr.* 32: 582, 1945.



portae at its entrance in the liver, in vena cava, distally to the vena hepatica, and in some cases also in venae hepaticae. The time sequence of the punctions of the different vessels varied in different experiments. In many cases vena cava was punctured before as well as after the other vessels. This was done in order to make possible the calculation of *interpolated* values in those experiments in which the serum-iron level was rapidly changing. Serum iron analysis was performed according to the method of HEILMEYER and PLÖTNER, modified by VAHLQUIST (1941).

Table 1.

*Comparison of serum iron values in porta- and cava blood.  
Control tests.*

Experi- ment no.	Serum iron γ % Fasting value	Interval minutes	Serum iron after laparotomy γ %			Anesthesia
			v. cava	v. porta	v. porta- v. cava	
130.....	141	8	139	143	+ 4	Numal
131.....	258	15	237	224	— 13	Numal
1.....	218	20	220 <sup>1</sup>	223	+ 3	Numal
2.....	248	40	243 <sup>1</sup>	247	+ 4	Ether
23.....	163	44	164	177	+ 13	Numal
28 <sup>2</sup> .....	204	56	202	217	+ 15	Numal
M =	205	31	201	205	+ 4	

<sup>1</sup> Blood from right heart ventricle.

<sup>2</sup> Experimental occlusion of pylorus.

*Results.* In order to test the possible influence of anesthesia and of laparotomy on the serum iron level some control experiments were performed in fasting animals. It is obvious from the results given in table 1 that the serum iron level did not show any big fluctuations during the operation.

In all, 34 iron absorption tests were performed. The results in table 2 demonstrate that the serum iron level in the porta blood was higher than that of the cava blood in 32 out of 34 experiments. In some cases the difference did not exceed that observed in the control tests, but in 26 out of 34 experiments the difference was more pronounced, in 4 experiments even exceeding 100 γ% with a maximal value as high as + 214 γ%. In this last experiment the porta blood during absorption demonstrated the exceedingly high serum iron value of 742 γ%, whereas the corresponding value of cava blood was only 528 γ%. The mean value of the porta—cava differences for the whole material

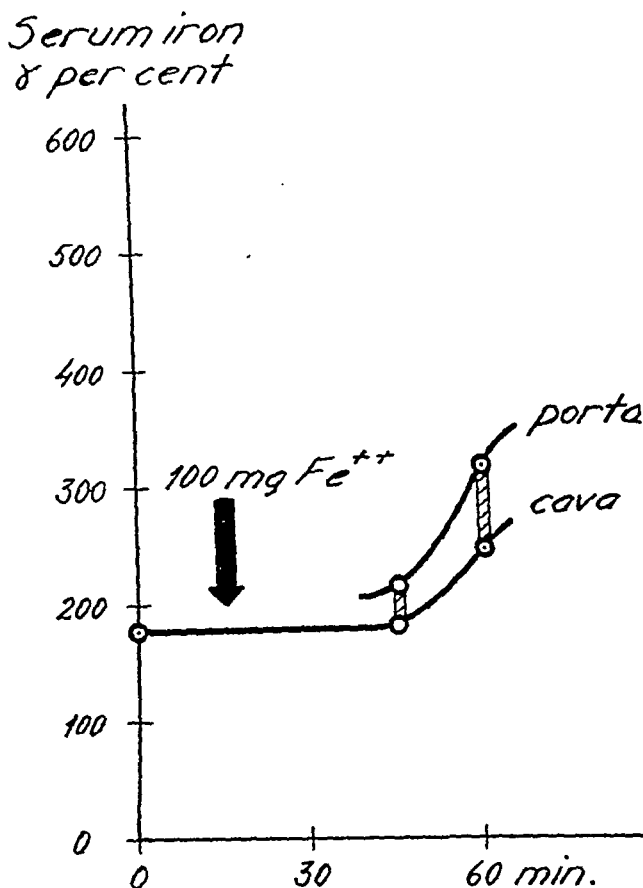


Fig. 1.

is  $46,9 \pm 8,1 \gamma\%$ , i. e. statistically quite significant. A typical experiment is given in figure 1.

The difference between the serum iron level of the porta and cava blood does not show any definite correlation to the intensity of absorption as it appears from the rise of serum iron in cava blood. In those cases where the difference surmounted 100  $\gamma\%$  the rise in cava blood was pronounced throughout, that is true. But otherwise the difference was sometimes marked also in cases in which the absorption was low or none as judged from the values of the cava blood (experiments number 34, 107, 109) and on the contrary there might be no difference at all also in cases in which the amount of the absorption was pronounced as judged from the serum iron rise of the cava blood (experiments number 13, 127, 32, 93). This last mentioned observation is remarkable and will be discussed later on.

Table 2.

Comparison of serum iron values in porta and cava blood during iron absorption tests (100 mg of iron administered by stomach tube).

Ex- peri- ment no.	Serum iron γ % Fasting value	Time after iron admin- istration minutes	Serum iron after iron administration			Type of serum iron curve at the investigation		
			rise in v. cava	rise in v. porta	v. porta- v. cava	in- crease	level	de- crease
6 <sup>1</sup>	224	25	+ 304	+ 518	+ 214	x		
102	134	14	+ 274	+ 406	+ 132	x		
17	188	44	+ 202	+ 334	+ 132		x	
4 <sup>1</sup>	276	45	+ 242	+ 360	+ 118			
111	293	33	+ 145	+ 242	+ 97			
95	187	50	+ 194	+ 276	+ 82		x	
34 <sup>2</sup>	176	44	+ 69	+ 141	+ 72		x	
105	215	32	+ 119	+ 190	+ 71	x	x	
94	200	39	+ 217	+ 282	+ 65		x	
18	292	57	+ 76	+ 139	+ 63		x	
103	203	22	+ 56	+ 114	+ 58	x		
107	242	32	+ 43	+ 100	+ 57	x		
24	144	44	+ 134	+ 181	+ 47	x		
12	147	27	+ 120	+ 164	+ 44			
109	340	28	+ 6	+ 42	+ 36	x		
27 <sup>2</sup>	208	38	+ 130	+ 164	+ 34		x	x
128	128	40	+ 281	+ 315	+ 34		x	x
8 <sup>3</sup>	233	50	+ 142	+ 174	+ 32	x	x	
108	258	37	+ 237	+ 265	+ 28		x	x
11	283	62	+ 109	+ 136	+ 27			
22	163	32	+ 167	+ 193	+ 26	x	x	
3 <sup>1</sup>	218	74	+ 118	+ 143	+ 25		x	
7	304	51	+ 37	+ 58	+ 21			x
35	153	138	+ 138	+ 158	+ 20			
26 <sup>2</sup>	117	55	+ 188	+ 207	+ 19		x	
14	236	26	+ 117	+ 134	+ 17		x	x
93	252	47	+ 172	+ 183	+ 11	x	x	
92	303	35	+ 59	+ 68	+ 9			x
19	248	40	+ 79	+ 87	+ 8			
13	136	33	+ 307	+ 312	+ 5	x		
104	264	23	+ 52	+ 54	+ 2	x		x
20	173	39	+ 156	+ 156	0		x	x
32	193	38	+ 177	+ 173	— 4	x		
127	99	37	+ 274	+ 266	— 8			
M =	212.6 ± 10.6	42.1 ± 3.6	+ 151.2 ± 14.0	+ 198.1 ± 18.3	+ 46.9 ± 8.1			

<sup>1</sup> Ether narcosis. <sup>2</sup> Experimental occlusion of pylorus. <sup>3</sup> 50 mg of ferrous iron.

In table 3 the total material given in table 2 is divided in two groups according to the size of the porta—cava difference of serum iron, and the results statistically treated. They throw light upon the point of view already discussed in the preceding section. They further indicate that the interval between iron

administration and collecting of the samples might influence the value of the porta—cava difference also if this interval ranged between 20—60 minutes. The difference is, however, in this material not statistically significant,  $12,0 \pm 7,0$  minutes.

A division of the material in two groups according to the fasting level of the serum iron gives the results collected in table 4. Obviously there is a tendency to more pronounced serum iron rise in the group with low fasting value. With respect to cava blood the difference in serum iron level in the two subgroups after iron administration is  $60,4 \pm 26,3$   $\gamma\%$ , *i. e.* statistically not significant, but it might perhaps have become so in a bigger material. As a consequence of the last mentioned observation the difference between the two groups with respect to serum iron concentration of porta and cava blood is less pronounced during iron absorption than in the fasting values. No relation was found between the size of the porta-cava difference and the fasting levels of the serum iron, the difference between the two groups being only  $4,6 \pm 16,5$   $\gamma\%$ .

From table 2 it is obvious that the porta—cava difference in serum iron concentration does not show any correlation to the tendency of the serum iron curve as judged from the values of the cava blood. Also in many experiments with declining serum-iron curve an excess of serum iron was noted in the porta blood. The difference between porta and cava blood in these cases was, however, always moderate and never exceeded 34  $\gamma\%$ .

In two experiments iron absorption tests were performed on animals which had been made anemic by bleeding (two bleedings within a week, about 60 cc in all). At the time of the absorption experiment the blood values of the two animals were as follows: No. 15: Hemoglobin 6.5 g%. Erythrocytes 2.0 mill. Serum iron 87  $\gamma\%$ . No. 16: Hemoglobin 7.7 g%. Erythrocytes 2.6 mill. Serum iron 53  $\gamma\%$ . The rise of serum iron in the cava blood in these experiments amounted to 313 and 394  $\gamma\%$  and hence was more pronounced than in anyone of the experiments in normal animals. Due to the rapid rise of the serum iron values the interpolated values of the bled animals is to some extent uncertain. Nevertheless it may be stated that the difference between porta and cava blood in these cases must have been very small if any (approximate values for no. 15: — 19  $\gamma\%$  and for no. 16: + 20  $\gamma\%$ ). Also the serum iron value of vena duodenalis in these cases corresponded to that of the vena porta.

**Table 3.**  
*The material in table 2 divided in two groups according to the height of the porta—cava differences.*

G r o u p	Number of experi-ments	Fasting values Mean	Interval after iron administra- tion Minutes	Rise in cava blood	Rise in porta blood	Difference between porta and cava blood
A: High values for porta—cava difference .....	17	211.6 $\pm$ 14.8	36.1 $\pm$ 2.7	+153.7 $\pm$ 21.8	+233.4 $\pm$ 30.2	79.7 $\pm$ 11.4
B: Low values for porta—cava difference .....	17	213.7 $\pm$ 15.6	48.1 $\pm$ 6.4	+148.8 $\pm$ 18.2	+162.8 $\pm$ 17.8	14.0 $\pm$ 3.0
Difference A—B:		—2.1 $\pm$ 21.4	—12.0 $\pm$ 7.0	+4.9 $\pm$ 28.4	+70.6 $\pm$ 35.1	+65.7 $\pm$ 11.8

**Table 4.**  
*The material in table 2 divided in two groups according to the height of the fasting values.*

G r o u p	Number of experi-ments	Fasting values		Interval after iron admin- istration Minutes	Rise in cava blood	Rise in porta blood	Difference between porta- and cava blood
		Limits	Mean				
C: High fasting values .....	17	215—340	263.6 $\pm$ 9.1	41.0 $\pm$ 3.5	+121.0 $\pm$ 19.6	+170.2 $\pm$ 29.7	49.2 $\pm$ 13.0
D: Low fasting values .....	17	99—208	161.7 $\pm$ 7.9	43.2 $\pm$ 6.4	+181.4 $\pm$ 17.6	+226.0 $\pm$ 20.1	44.6 $\pm$ 10.2
Difference C—D:		—	+101.9 $\pm$ 12.1	—2.2 $\pm$ 7.3	—60.4 $\pm$ 26.3	—55.8 $\pm$ 35.8	+4.6 $\pm$ 16.5

Table 5.

*Serum iron values in different vessels during iron absorption tests (100 mg of iron administered with stomach tube).*

Experi- ment no.	Serum iron % Fasting value	Time after iron adminis- tration minutes	Serum iron in cava blood after iron adminis- tration	Serum iron values in other vessels compared with the values of cava blood		
				v. duode- nalis	v. porta	v. hepa- tica
34	176	43	245	—	+ 72	+ 29
24	144	43	278	+ 55	+ 47	—
12	147	30	267	+ 83	+ 44	+ 19
128	128	40	409	—	+ 34	+ 40
8 <sup>1</sup>	233	55	375	+ 60	+ 32	+ 33
11	283	65	392	—	+ 27	+ 40
22	163	33	330	—	+ 26	+ 16
7	304	55	341	— 4	+ 21	+ 14
35	153	137	291	—	+ 20	+ 22
14	236	30	353	—	+ 17	+ 41
32	193	30	370	—	— 4	± 0
M =	196	51	332	(+ 49)	+ 31	+ 25

<sup>1</sup> 50 mg of iron.

In some experiments puncture was performed also in other vessels than vena cava and vena porta. The results are gathered in table 5. The number of experiments is too small to permit any valid conclusions. It is, however, obvious that the serum iron level sometimes is higher in vena duodenalis than in vena porta (experiment no. 8 and 12). Concerning vena porta and vena hepatica a positive difference with higher values in vena porta could be demonstrated only twice (experiments no. 12 and 34). In one experiment (no. 14) the blood leaving the liver had a higher serum iron level than that entering the organ. This observation will be discussed below.

In a small series of comparison amino acid absorption tests were performed followed by amino acid determination (method KRAUEL) of porta and cava blood. The results are collected in table 6. The number of experiments is rather scanty but they seem to indicate that the porta—cava difference is more pronounced for the amino acids than for the serum iron. Evaluating these results it must be recognized that the type of the absorption curves depends upon the balance between inflow in the blood and outflow in the tissues. Intravenous injection experiments with iron

Table 6.

Comparison of amino acid nitrogen concentration in porta- and cava blood during amino acid absorption test (10 gram of casein hydrolysate ("Aminosol") given by stomach tube)).

Experiment no.	Amino acid conc. mg% Fasting value	Time after administration minutes	Amino acid conc. mg%, after per oral amino acid administration			Type of amino acid curve		
			Rise in cava blood	Rise in porta blood	Porta—cava blood	in-crease	level	de-crease
155	8.85	63	+ 4.91	+ 9.67	+ 4.76	x	x	
150	9.35	75	+ 7.20	+ 11.47	+ 4.27		x	
151	9.92	48	+ 8.26	+ 11.90	+ 3.64		x	
153	7.94	44	+ 4.84	+ 7.32	+ 2.48		x	
154	10.55	55	+ 7.70	+ 8.85	+ 1.15		x	
152	8.80	23	+ 2.78	+ 3.72	+ 0.94	x	x	
M=	9.22	51	+ 5.95	+ 8.82	+ 2.88			

show that the iron disappears rather slowly in the tissues (cf. NILSSON, 1948). As for the amino acids obviously the reverse is true, these substances leaving the blood most rapidly (VAN SLYKE and MEYER 1913—14).

*Discussion.* The literature is remarkably scanty as to observations concerning the chemical composition of the porta blood during absorption from the digestive tract. We have been able to find only some notes concerning the blood sugar values in these circumstances. In dogs which had been angiotomated in vena porta and other vessels according to the method of London some authors have observed higher blood sugar values in vena porta than in other vessels (KOTSCHOFF 1927; CHERRY and CRANDALL Jr. 1937). The differences observed have been rather varying in size.

The following mean values can be collected from one of the tables in the paper of CHERRY and CRANDALL (the results are based on 6 experiments, limit values given in brackets): fasting value 87 mg% (73—94 mg%); blood sugar rise in arteria femoralis 30 minutes after administration of 20 g of glucose, 24 mg% (5—41 mg%); difference porta—aorta 24 mg% (0—39 mg%); difference porta—hepatica 17 mg% (—7—39 mg%).

In judging the results just mentioned due respect must be given to the fact that the liver is supplied with blood from two different sources, vena porta and arteria hepatica. Recent in-

vestigations demonstrate (GRINDLAY et al. 1941; LIPSCOMB and CRANDALL 1947) that in dogs arteria hepatica supplies about  $\frac{1}{4}$  of the total blood inflow in the liver, vena porta about  $\frac{3}{4}$ . The quotient is, however, materially changing and easily influenced by anesthesia and surgical interventions.

The amount of iron administered was in our experiments comparatively high, corresponding to about 30—40 mg per kg of body weight. As a matter of fact only a small part of this amount could reach immediate contact with the mucosa of the stomach and the gut. Even after 24 hours' starvation the stomach of the rabbit contains big amounts of ingesta. And furthermore the pyrophosphate compound represents an iron salt which is comparatively well tolerated. In a study on children on the digestive disturbances during iron therapy (D'AVIGNON and VAHLQUIST, 1948) it was found that the patients tolerated several iron compounds, among them pyrophosphate, without severe toxic symptoms also in a dosage of 45 mg of iron per kg of body weight and day.

The difference in serum iron concentration between porta and cava blood during iron absorption in our experiments varied between  $-8 \gamma\%$  and  $+214 \gamma\%$  with an average value of  $+46.9 \pm 8.1 \gamma\%$ . The difference is statistically significant and comparable to that observed in the glucose absorption tests. The experiments with amino acid absorption shortly related above demonstrate a difference in amino acid concentration between porta and cava blood which is more pronounced than in the case of iron and sugar. This fact might be explained by the rapid outflow of amino acids in the tissues and to some extent perhaps excretion with the urine.

A difference in serum iron concentration between vena porta and vena cava blood may be caused by retention of iron in the liver. But admittedly it might be to some extent explained also as a phenomenon of dilution. A rapid rise of the serum iron concentration in the blood of the peripheric vessels is possible only provided that the serum iron concentration of the blood from the areas of absorption is definitely in excess. A simple calculation will elucidate this fact.

The serum iron increase per minute in cava blood, measured as the  $\frac{\text{total observed serum iron increase}}{\text{time interval from iron administration}}$ , amounted to a maximal value of about  $10 \gamma\%$ . Provided the time of circula-



tion for the total blood volume in rabbits is taken as 20 seconds and further on that 20 % of the total blood volume is found in the porta system a rise of 10  $\gamma\%$  per minute in cava blood could be found only if the mean excess of the serum iron concentration of porta blood is  $\frac{20}{60} \times \frac{100}{20} \times 10 = 15-20 \gamma\%$ . In the blood from the absorption areas proper the excess must be even greater.

The calculation given above may of course be criticized. The values of circulation time and relative blood volume in the porta system are approximate. As far as the former one is shorter and the later bigger the serum iron in porta must not be so big as mentioned. But on the other side it seems clear that the inflow of iron with the porta blood in some phases of absorption must have been more pronounced than the average value of about 10  $\gamma\%$ /minute. Even if the outflow of iron from the blood into the tissues is a relatively slow process, as indicated by experiments with intravenous iron administration, it is nevertheless obvious that such an outflow does exist already from an early stage of the absorption. And, furthermore, due respect must be given to the fact that there is always a lapse of time until the iron reaches extensive contact with the mucosa and the absorption is on a maximal level.

If one wants to study the immediate influence of the liver on the iron absorption, the most exact method is of course the comparison of the serum iron level in the inflowing porta blood and the outflowing hepatica blood. A slight decrease, corresponding to about  $\frac{1}{4}$  of the porta—cava difference, may be explained by the mixing of blood from arteria hepatica but bigger differences than that could be explained only by real iron retention in the liver.

Unfortunately it is rather difficult to puncture the short, deep lying venae hepaticae. Technically satisfying punctures could be performed in ten experiments. In eight of these with a porta—cava difference varying between  $-4$  and  $+34 \gamma\%$ , the porta—hepatica difference varied between  $+10$  and  $-24 \gamma\%$ . The lowest value of  $-24 \gamma\%$  is remarkable. It was obtained in an experiment where double analyses were done throughout. The result should indicate that the liver might sometimes give away iron already during actual absorption from the digestive tract. As a single result it should be, however, judged with caution.

In the two experiments with highest values of porta—cava difference, + 44 and + 72  $\gamma\%$  of this special series, the porta—hepatica difference was + 25 and + 43  $\gamma\%$ . Only these two experiments demonstrate directly a retention of iron in the liver.

In six experiments the porta—cava difference surmounted the value of + 72  $\gamma\%$ , but puncture of the *venae hepaticae* was not performed in these cases. It seems, however, quite unquestionable that a porta—cava difference exceeding a value of say 100  $\gamma\%$  could not be explained otherwise than by retention of iron in the liver. And even more, it is probable that such a retention must have taken place as soon as the porta—cava difference exceeds some 20  $\gamma\%$ , as far as the calculation given above is accepted and the outflow of iron in other tissues than the liver has not been abnormally increased. Even a porta—cava difference lower than 20  $\gamma\%$  might sometimes be an indicator of retention of iron in the liver, namely in those cases where the degree of iron absorption as measured from the serum iron rise in the cava blood, was moderate or low.

There is a parallelism between the observations here discussed and the results of recent studies by MOORE's team (1948). In experiments on human beings they found a retention of iron in the tissues which in non-anemic individuals was not less than that on an average found in the hemoglobin of the erythrocytes.

The results of iron absorption tests in animals made anemic by acute hemorrhage were queer. It was an expected finding that the initial low serum iron values should rise more than in any of the experiments with normal animals, as it is a well known fact that iron deficiency enhances iron absorption. But it is a surprising observation that the values of the porta—cava difference should be zero in both experiments. Admitted that the demand for iron of the liver during prevailing circumstances could be small to secure the priority of other tissues, especially the bone marrow, it is nevertheless remarkable that even the expected slight excess of serum iron in the *vena porta* due to iron absorption was not found. It is difficult to give a satisfactory explanation of this finding. According to our opinion the possibility must be taken under consideration that the iron might have found abnormal pathways for absorption, *i. e.* the lymphatic channels. According to MOORE et al. (1939) the absorption of iron should proceed solely with the porta blood. This opinion is, however, based on the results of a single experiment in a dog.

In the blood of the vena duodenalis, serum iron values were sometimes observed which were definitely higher than those of the porta blood. This observation is quite natural realizing the fact that the blood in the porta vein to a large extent emanates from sections of the gut, which could definitely not be the seat of iron absorption.

*Conclusion.* The serum iron level of the porta blood of rabbits during iron absorption is in most cases definitely higher than that of the cava blood. The difference varies individually, sometimes being pronounced with values as high as over 200  $\gamma\%$ . To some extent this difference may be explained solely by the inflow of iron from the stomach and the gut. In many cases the difference is, however, of such a size that it could be explained only by iron retention in the liver. In some cases such an effect could be demonstrated directly by means of determining separately the serum-iron content of the blood in the vena porta and the vena hepatica.

### Summary.

1. Iron absorption tests were performed in rabbits. In 34 experiments 100 mg of ferrous iron was administered orally.

2. 20—60 minutes after iron administration the animals were laparotomized in Numal narcosis. Puncture of vena porta and vena cava, in a small group of experiments also vena hepatica and vena duodenalis, was performed.

3. In 32 experiments out of 34 the serum iron level of the porta blood was higher than that of the cava blood. The difference varied between  $-8$  and  $+214 \gamma\%$ , with an average value of  $+46,9 \pm 8,1 \gamma\%$ .

4. The high serum iron level of the porta blood during iron absorption reflects the enrichment with iron during the passage through the digestive tract. To some extent the difference between porta and cava blood could be explained by the fact that it is necessary in order to heighten the serum iron level of the total blood volume. But the big differences often observed could be explained only by the assumption that iron is taken up by the liver from the porta blood. In some experiments the retention of iron in the liver could be directly demonstrated by means of comparison of the serum iron level of the porta blood and the hepatica blood.

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## Studies on the Absorption of Iron.<sup>1</sup>

### III. The Serum Iron Curve in Rabbits Following Intravenous Administration of Iron in Massive Doses.

By

GÖSTA NEANDER and BO VAHLQUIST.

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Several investigators have studied the serum iron curve following the administration of iron intravenously in human beings (SKOUGE 1939, BRÖCHNER-MORTENSSSEN 1943, WALDENSTRÖM 1944). Healthy adults tolerate on an average 0.15 milligrams per kilogram of body weight without toxic symptoms. In Waldenström's series the rise in serum iron observed five minutes after the injection of 10 milligrams of ferric iron amounted to about 200  $\gamma\%$  corresponding to some 60 per cent of the rise expected with reference to the total plasma volume. The following decline of the serum iron curve was very slow, and even after two hours the serum iron level was not definitely lower than that initially obtained.

During disease the serum iron curve following intravenous injection of iron is often materially changed. In iron deficiency states and during infections there is a more rapid decline from the values initially obtained, probably as an expression of increased avidity for iron in the body tissues.

In pernicious anaemia the initial serum iron increase is, as a rule, essentially lower than that expected. This peculiar "braking effect" (WALDENSTRÖM) was later on explained by the discovery

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<sup>1</sup> Part II of this series: "Experimental studies on the serum iron level in the porta vein during iron absorption tests" is published in *Acta physiol. Scand.*: 1949.

of a special iron carrying protein fraction of the blood plasma, the saturation limit of which was settled at 3—400  $\gamma\%$  (HOLMBERG and LAURELL 1945). In healthy adults this specific fraction is saturated only to some 50 per cent of its iron carrying capacity. In pernicious anaemia with hypersideraemia the margin between actual serum iron concentration and upper saturation limit is often narrow and hence only small quantities of iron could be given before the saturation limit is passed and toxic symptoms appear.

In animal experiments we have studied the serum iron curve following the intravenous injection of *massive* doses of iron, usually corresponding to 0.5—2.0 mg per kilogram of body weight. The toxic symptoms sometimes observed were carefully noted.

*Material and technique.* White rabbits weighing about 2.0—2.5 kilograms were used as experimental animals. Iron ammonium citrate (ferric iron) was given in doses usually varying in iron content between 0.5 and 2.0 milligrams per kilogram of body weight. A stock solution with 2.0 milligrams iron per cubic centimetre was diluted with normal saline according to the needs and the final volume adjusted to about 4 cubic centimetres. The duration of the injection usually varied from 2 to 4 minutes. The first bleeding and the injection of the iron was done in the auricular vein of one side and the following bleedings in the ear of the other side. After administration of massive doses a tendency to constriction of the vessels was observed, sometimes so severe as to necessitate heart puncture for obtaining the blood. Serum iron analysis was performed according to the method of Heilmeyer and Plötner with the modifications of Vahlquist (1941).

*Results.* The total material is presented in table 1.

In a previous investigation one of the authors (VAHLQUIST) found the mean value of fasting serum iron values in rabbits to be 182  $\gamma\%$ . In the present investigation the mean value was somewhat higher, 230  $\gamma\%$ , with observed limit values of 132 and 354  $\gamma\%$ .

When analysing the blood immediately (0.5—2 minutes) after completed iron injection the increase of serum iron observed never exceeded 30 % of that calculated, and the mean value was  $22.6 \pm 1.2$  %. The percentage figures given are calculated from a hypothetical 100 % value which should be obtained if the total of the iron remained in the blood plasma and if the value of the blood plasma could be taken as 4.5 % of the body weight.

The values collected in table 1 do not support any idea of interdependence between the degree of serum iron rise and the serum iron fasting levels.

Table  
*Serum Iron Values in Rabbits Following*

Group	Animal no.	Amount of iron inj. Fe + + + mg/kg	Serum iron initial level $\gamma$ %	Serum iron	
				0.5—2 min.	
				$\gamma$ %	per cent of calculated initial rise
A	120	2.0	217	+ 1,181	27
	119	»	250	+ 1,016	23
	140	» <sup>1</sup>	210	+ 810	18
	118	»	257	+ 685	15
	117	1.8	236		
	116	»	354		
	115	»	221		
	114	»	199		
	113	»	190	+ 878	22
	112	»	208		
	60	»	166		
	59	1.5 <sup>2</sup>	196		
	137	»	283	+ 545	16
	61	» <sup>3</sup>	244		
Mean =			230.8 $\pm$ 12.5	+ 853	20.2 $\pm$ 1.9
B	123	1.0	288	+ 664	30
	139	» <sup>1</sup>	291		
	122	»	221	+ 507	23
	121	»	204	+ 59	27
	135	»	260		
	58	0.7	132	+ 332	21
	56	»	336	+ 284	18
	126	0.5	228	+ 269	24
	125	»	152	+ 304	27
	124	»	205	+ 279	25
	57	» <sup>3</sup>	208		
Mean =			229.5 $\pm$ 18.3	+ 404	24.4 $\pm$ 1.4
Difference A—B					4.2 $\pm$ 2.4
Total material (A + B)			230.2 $\pm$ 10.4		22.6 $\pm$ 1.2

<sup>1</sup> Fe++ as ferrous sulphate.<sup>2</sup> 10 min. after iron injection.<sup>3</sup> 1.58 mg Fe + + + /kg.

The increase of the serum iron roughly parallels the amount of iron injected during the premises given in these experiments, i. e. with an amount of iron corresponding to 0.5—2.0 mg per

1.

*Intravenous Injection of Iron in Massive Doses.*

increase after					
5—6 min.		60—75 min.		240—300 min.	
$\gamma$ %	per cent of calculated initial rise	$\gamma$ %	per cent of calculated initial rise	$\gamma$ %	per cent of calculated initial rise
+ 682	15	+ 297	7		
		+ 258	6		
		+ 236	5		
		+ 179	4		
+ 652	16	+ 244	6	+ 166	4
+ 576	14	+ 132	3	+ 2	0
+ 421	11	+ 263	7	+ 163	4
+ 299	7	+ 187	5	+ 101	3
+ 800 <sup>a</sup>	20				
+ 587	15	+ 359	9	+ 272	7
+ 952	27	+ 546	16	+ 218	6
		+ 249	7		
+ 862	27	+ 398	12		
+ 648	16.9 $\pm$ 2.1	+ 279	7.3 $\pm$ 1.0	+ 154	4.0 $\pm$ 1.0
+ 402	18	+ 162	7		
+ 390 <sup>a</sup>	18	+ 118	5		
+ 383	17	+ 261	12		
		+ 236	11		
+ 340	15	+ 75	3		
		+ 220	14	+ 68	4
+ 240	22	+ 146	9	+ 38	2
+ 276	25	+ 160	14		
+ 235	21	+ 214	19		
+ 300	34	+ 181	16		
+ 321	21.3 $\pm$ 2.2	+ 177	11.0 $\pm$ 1.6	+ 53	3.0 $\pm$ 1.0
	4.4 $\pm$ 3.0		3.7 $\pm$ 1.9		
	18.9 $\pm$ 1.6		8.95 $\pm$ 1.0		3.8 $\pm$ 0.8

<sup>a</sup> 1.46 mg Fe +++/kg.<sup>b</sup> 0.4 mg Fe +++/kg.

kilogram of body weight. A division of the material in two groups according to the amount of iron injected gives an average serum iron increase of 24.4 % of that calculated in the group 0.5—1.0 mg



Table 2.

*Signs of Intoxication in Rabbits After Intravenous Injection of Iron in Massive Doses.*

Animal no.	Weight	Amount of iron injected, mg/kg	Serum iron			Signs of intoxication
			Before	After		
			γ %	Min.	γ %	
82	2.7	62.0	—	—	—	Injection of 31 mg/kg two times with 7 minutes' interval. After the first injection dyspnoea and tachycardia. Evacuation of urine and stool. After the second injection opistotonus, twitching, coma and death within 3 minutes.
81	1.8	10.2	247	4	5,850	Injection of 0.9 mg/kg and 9.3 mg/kg with 5 minutes' interval. Restless during the second injection but no signs of paralysis.
80	2.4	8.4	270	8	3,470	Injection of 1.4 + 7.0 mg/kg with 10 minutes' interval. After the first injection paralysis of the lower limbs. After the second injection paralysis extended to the caudal part of the trunk and further on tachycardia, diarrhoea, twitching, dyspnoea and hypotonia developed.
120	2.3	2.0	217	1½	1,398	At the end of the injection convulsions and opistotonus. Later on paralysis of the lower half of the body.
119	2.3	2.0	250	½	1,266	No signs of intoxication.
118	2.5	2.0	257	1½	942	No signs of intoxication.
60	2.3	1.7	166	6	753	After the injection paralysis of the lower limbs.
59	1.9	1.5 <sup>1</sup>	196	6	1,148	No signs of intoxication.
61	2.4	1.5 <sup>2</sup>	244	6	1,108	During the injection convulsions, opistotonus. Afterwards completely paralysed in the lower limbs.

<sup>1</sup> 1.58 mg/kg. — <sup>2</sup> 1.46 mg/kg.

Animal no.	Weight	Amount of iron injected, mg/kg	Serum iron			Signs of intoxication
			Before	After		
			γ %	Min.	γ %	
123	2.3	1.0	288	1	952	No signs of intoxication.
122	2.2	1.0	221	1/2	728	No signs of intoxication.
121	2.5	1.0	204	1/2	796	Restless during the injection. No convulsions. Afterwards signs of paralysis in the lower limbs.
58	1.7	0.7	132	5	464	Somewhat uneasy after the injection. No local signs of intoxication.
56	2.4	0.7	336	3	620	No signs of intoxication.
124	2.5	0.5	205	2	484	No signs of intoxication.
57	2.0	0.4	208	5	508	No signs of intoxication.
55	2.6	0.35	207	16 1/2	475	No signs of intoxication.
54	2.2	0.18	189	17	327	No signs of intoxication.

per kilogram of body weight, and 20.2 % of that calculated in the group 1.5—2.0 mg per kilogram of body weight. The difference is by no means statistically significant,  $4 \pm 2.4$  %.

The course of the serum iron curve during the first hour after the iron injection varies individually but is on the whole declining. Without exception the serum iron values after 60—75 minutes are, however, above the initial level. The difference varies between 75 and 546  $\gamma$ % with an average of 232.9  $\gamma$ %. In absolute figures the elevation is more marked in the group with 1.5—2.0 mg of iron per kilogram of body weight with an average of 279.2  $\gamma$ % as compared with + 177.3  $\gamma$ % in the group with 0.5—1.0 mg of iron. Calculated as per cent the remaining increase is somewhat more pronounced in the group with the lowest iron amount, 0.5 mg per kilogram of body weight, but the number of experiments is not large enough to permit any definite conclusions. For the total material the serum iron excess after 60—75 minutes corresponds to  $8.95 \pm 1.0$  % of the calculated initial increase.

In seven animals the serum iron level was determined also four or five hours after the iron injection. In six of these there was at that time still a moderate increase as compared with the initial values.

The toxic reactions following the iron injection were, of course, more pronounced the larger the amount of iron injected. Still

the individual variations were marked in this respect too, as is evident from the results collected in table 2.

The results in table 2 show that serious reactions with paralysis once occurred already with 1.0 mg of iron per kilogram of body weight whereas some of the other animals tolerated 2.0 mg of iron without obvious toxic reaction. Curiously enough one animal (nr 81) showed only minor signs of intoxication after a huge iron dose of 10.2 mg per kilogram of body weight. With iron doses corresponding to or below 0.5 mg toxic signs were never observed.

The toxic signs noted in various experiments were especially the following ones: restlessness, dyspnoea, twitchings, convulsions, paralysis, opisthotonus, evacuation of urine and stool, exitus.

*Discussion.* It seems to us probable that the behaviour of the serum iron after injection of massive iron doses might be explained in the following way. In animals like in human beings there is in the blood a special iron carrying protein fraction, in rabbits corresponding to some 300  $\gamma\%$  (LAURELL, unpublished). As soon as the saturation limit of this fraction is passed the iron is no longer retained in the blood but leaks out in the extracellular tissue fluid. The iron content of this fluid is during normal conditions essentially lower than that of the plasma. In the experiments of MOORE et al. (1939), they found the relation between lymph iron and serum iron in a dog to be about 1:2. The total volume of the extracellular tissue fluid is assumed to be three times that of the blood plasma. If iron is injected in large amounts it will be distributed between the blood plasma and the lymph probably in an equilibrium of the Donnan type.

The amount of iron recovered from the plasma as judged from the serum iron increase varied individually a good deal. To some extent this fact might be explained by individual variations in blood plasma volume. This volume sometimes being materially higher or lower than the mean of 4.5 per cent used in the calculations. It is, however, hardly possible, that the big differences sometimes observed, *i. e.* from 1,181  $\gamma\%$  to 685  $\gamma\%$  in four experiments with 2.0 mg of iron per kilogram of body weight, could be explained solely in this way. The cause remains obscure but it should be noted that similar observations have been made also after the injection of lower iron doses in human beings (BRÖCHNER-MORTENSSSEN, 1943).

The fact was mentioned above that even if iron is injected in quantities probably not exceeding the iron carrying capacity of

the serum, on an average only about 60 % of the calculated rise in serum iron is obtained. In our experiments with massive iron doses the recovery was much less than that, on an average only 22.6 %. It seems reasonable to assume that this immediate "disappearance" of the mass of iron injected could be explained as essentially due to a distribution over the total of the extracellular fluid. The ensuing decline of the serum iron, which in all probability reflects a similar decline of the iron content of the extracellular tissue fluid, might be explained by an uptake of iron in the tissue cells, especially perhaps in those belonging to the reticulo-endothelial system, and furthermore to excretion with the urine. During physiological conditions there is hardly any excretion of iron in the urine. In pronounced hypersideremia, however, such a possibility must be taken under consideration.

The decline of the serum iron is most rapid during the period immediately following the injection. Gradually it then subsides, the complete return to the initial level usually not being obtained until four or five hours after the injection or later. In absolute figures the rate of decline is more rapid the higher the initial serum iron level obtained after the iron injection. But in relative figures the rate of decline is roughly constant within the limits given in our experiments.

The iron salt used in our study was as a rule the ammonium citrate, *i. e.* the iron was in the ferric form. In two experiments we resorted to the use of ferrous iron, in the form of ferrous sulphate.<sup>1</sup> The results of these latter experiments did not differ from the other ones as to the behaviour of the serum iron. Nor did they indicate that the iron in the ferrous state should produce toxic reactions more easily than the ferric iron.

The manner of linkage of the iron in the compound used is of deciding importance for the result of the intravenous injection. If the iron is coupled in a non dialyzable form, such a compound might be given without harm also to human beings in a dosage exceeding 1 mg per kilogram of body weight (LINDGREN and AGNER, 1945). The observations of these authors clearly demonstrate that the toxic reactions otherwise observed have nothing to do with the serum iron level *per se* — in one of the experiments of LINDGREN and AGNER a serum iron level of 1,840  $\gamma\%$  was noted — but appear only if there is a flooding of iron in the tissues or at

<sup>1</sup> The iron preparations used for injection were kindly prepared by Pharmacia Ltd. The authors express their sincere thanks for their cooperation.

least the iron is offered to the endothelial cells of the capillaries in an easily dialyzable form.

The toxicology of iron ought to be carefully reviewed once again in the light of newer knowledge concerning the metabolism of that metal. It is an interesting observation that acute poisoning due to iron may be achieved not only by intravenous injection of compounds of the metal but also after per oral intake of heavy amounts of compounds frequently in use. Recently several cases of fatal poisoning in children due to massive intake of tablets of ferrous sulphate or ferrous chloride have been reported (FORBES, 1947, THOMSON, 1947, BELLINDER, 1948, LINDQUIST, 1948).

The results of animal experiments collected in table 2 demonstrate that there are pronounced individual variations in the sensibility to iron. On the whole the toxic signs appear only at higher concentration in animals (obviously to some extent depending on the fact that the most early ones are of purely subjective nature, such as nausea, retrosternal pressure, itching of the nose). Otherwise pronounced signs of toxic influence could be observed in one animal with 0.7 mg of iron per kilogram of body weight, whereas other animals tolerated up to 2.0 mg without obvious toxic signs. To conclude we believe that the same individual variations observed in laboratory animals exist in humans.

### Summary.

1. Iron in the form of ferric ammonium citrate was administered intravenously to rabbits in doses of 0.5—2.0 mg per kilogram of body weight.

2. The serum iron values observed within two minutes after the injection never exceeded 30 per cent of those calculated. In many cases they were materially lower than that. The following course of the serum iron curve showed a successive decline. After four hours the serum iron level still was above the initial fasting level.

3. The "disappearance" of 70 to 80 per cent of the iron, already during the time of injection, is probably the result of a rapid distribution between the blood plasma and the extracellular tissue fluid. The following successive decline is probably explained by iron absorption of the tissue cells, to some extent perhaps also by excretion in the urine.

4. The toxic signs following the injection of iron intravenously in relatively large doses are reported. The individual variations were pronounced.

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From the Histological Department of the University, Lund.

## The Functional Organization of the Peripheral Autonomic Innervation.<sup>1</sup>

By

NILS-ÅKE HILLARP.

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Numerous, sometimes highly divergent views have been advanced concerning the structure of the peripheral autonomic innervation. A critical survey of these views has been published by the author in a previous paper (HILLARP, 1946), presenting at the same time neuro-histological investigations, which led to a new conception of this problem.

According to this view the innervation of autonomic effector cells takes place by means of a nervous ground plexus: A plexus of axons running in a fine-meshed network of anastomosing strands formed by the terminal Schwann plasmodium. All the effector cells are probably in direct contact with this ground plexus. The assumption that this nervous structure is the actual peripheral innervation apparatus is partly hypothetical, however. It is based on the view that the ground plexus is a closed terminal formation. Whether it really is so, cannot be determined with the neurohistological methods available at present. The entire construction of the nervous ground plexus speaks so strongly in favour of this conception, however, that we can be justified in accepting it — a least provisionally.

However, it is hardly possible to clear up the functional organization of the peripheral autonomic innervation exclusively with neurohistological technique. The construction of the ground

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<sup>1</sup> Aided by a grant from the Faculty of Medicine, University, Lund.

plexus does not permit an analysis of the extension of the individual neurone within it. It would therefore be too rash to attempt an evaluation of the spreading of a nervous impulse within the plexus on the basis of its morphology alone. This spreading may be assumed to take place either altogether diffusely, or to be more or less limited locally. The response of the effector system to a nervous stimulus from an individual post-ganglionic nerve fiber may consequently also be either diffuse or localized.

There is at present only one logically conceived theory regarding the functional organization of the innervation of autonomic effector systems. This theory, launched by CANNON, ROSENBLUETH and collab. (See ROSENBLUETH, 1932, 1936, ROSENBLUETH and RIOCH, 1933, CANNON and ROSENBLUETH, 1937) is founded on the assumption that only some of the effector cells are directly innervated. In or at these innervated cells — "key cells" — the chemical mediator is liberated on stimulation and then diffuses to the non-innervated cells. If this diffusion were limited to a certain number of effector cells, some units resembling the motor units in the skeletal musculature might be conceived to exist in the smooth musculature, too. If on the other hand the diffusion were not limited locally but instead quite diffuse, the effector system would always react as a whole even if only a fraction of the nerves were stimulated. According to ROSENBLUETH and RIOCH the latter alternative is indeed the correct one.

Because of the difficulties in recording in an autonomic effector system the reaction of a single cell upon a nervous stimulus, the innervation problems have generally been attacked by indirect methods. However it was possible to read off directly the cell reaction in the adrenal medulla on stimulation of its nervous centres (HILLARP, 1946). A closer analysis of the experiments described in that paper resulted in the following conception of the functional organization of the peripheral autonomic innervation, which may give an alternative explanation of the observations made by ROSENBLUETH and collab.

*The peripheral innervation apparatus consists of the nervous ground plexus, formed by a terminal Schwann plasmodium, within which terminal axon ramifications are running. Within the ground plexus each axon has a certain extension, and innervates in its course a certain number of cells, which react as a functional unit, the neuro-effector unit. This unit is not innervated by one neurone*



alone, however, but several neurones converge towards it, neurones whose terminal axon ramifications run within the same strands of the ground plexus. By the overlap thus present in the innervation structure proper the response of the autonomic effector system on indirect stimulation may be modified both by temporal and by spatial summation effects.

It may however be questionable whether or not the adrenal medulla has a special position due to its praeganglionic innervation. It is therefore an open question whether the interpretation of the functional organization of the innervation in the adrenal medulla may be generalized to hold for other autonomic effector systems, too. Thus it seemed necessary to extend our investigations. In this paper experiments on salivary glands as well with adrenergic as with cholinergic innervation are reported.

### Material and Methods.

The experiments were carried out on a number of about 100 albino rats, aged 2—3 months.

The reactions of the glandular cells upon indirect stimulation were investigated in the submaxillary and sublingual glands as follows:

The glands were totally or partially denervated on the left side while the strength of stimulation was controlled on the glands of the right side possessing intact innervation. The glandular cells have been thrown into an intensive secretion by giving the animals dry starch for 30—60 minutes after a previous starvation for 24—48 hours (provided only with water ad lib.). Immediately afterwards the animals have been killed by means of a blow on the neck and the glands fixed by injecting Susa solution via the carotid arteries. Embedded in paraffin, serial 5  $\mu$  sections were prepared of the glands and every tenth section stained with Azan. This staining technique proved to be favourable for the differentiation between inactive and active glandular cells.

Total denervation was carried out by extirpation of the chorda tympani in the middle ear and by extirpation of the cervical sympathetic trunk down to the subclavian artery. Partial denervation was carried out in three ways:

- 1) Partial transection of the cervical sympathetic trunk (about 1 cm. caudally from the cranial cervical ganglion) by cauterization with a diathermal microelectrode.

- 2) Partial cauterization by diathermy of the submaxillary ganglion in the hilus of the gland.

- 3) Partial cauterization by diathermy of the nerves round the submaxillary artery immediately before its entrance into the gland.

All the operations were carried out 10 days before the start of the experiments.

## Results.

### 1. Submaxillary gland.

As a first step the stimulation technique described above has been studied in order to establish the registrable cell changes either in glands possessing intact innervation or in cases of total sympathetic or parasympathetic denervation. — All the terminal portions are constituted of a single cell type of serous character.

The applied stimulation evokes great cytological changes in the glandular cells with intact innervation:<sup>1</sup> the acini are markedly shrunk, the cytoplasm of the cells is often vacuolated and more less stained with azocarmine. Even the nuclei have a characteristic appearance: they are big and round, having a fine-meshed chromatine network and a prominent nucleolus.

After a total sympathetic denervation of the gland the same stimulation does not evoke the changes mentioned above: in this case the terminal portions are voluminous, the cytoplasm stainable with aniline blue, and not at all vacuolated. The nuclei lacking a distinct nucleolus, are compact and strongly stainable.

This picture of relative inactivity is still more accentuated in case of a combined sympathetic-parasympathetic denervation. The appearance of the glandular cells here is highly suggestive of the classical picture of mucous cells. On the other hand after merely a chordectomy, the intact sympathetic innervation induces such a high activation of the cells that the acini become small and shrunk. However, no cytoplasm vacuolization can be observed and no prominent nucleolus is present in the nuclei.

*Thus the applied stimulation technique induces such great and characteristic cytological changes in the gland, that it enables us to differentiate denervated cells and cell complexes from those possessing intact innervation.*

In the principal series the experimental conditions were identical with those mentioned above, differing only in the mode of denervation: namely the glands on the left side were exposed only to a partial denervation instead of a total sympathetic or parasympathetic denervation.

After a partial cauterization of the cervical sympathetic trunk

<sup>1</sup> Microphotographs of the cytological picture on stimulation of the salivary glands as well with intact innervation as after the various types of denervation will be published in a paper on the innervation of the salivary glands (HILLARP, 1948).

the glands show a varied appearance according to the grade of denervation. In some cases practically the whole parenchyme shows the typical picture of a sympathetic denervation, while in other cases nearly the whole innervation seems to be intact. In a number of rats, however, the histological appearance of the submaxillary gland is dominated by two -- cytological quite different -- types of acini. The first type has small, shrunken cells with often vacuolized cytoplasm, stainable with azocarmine and

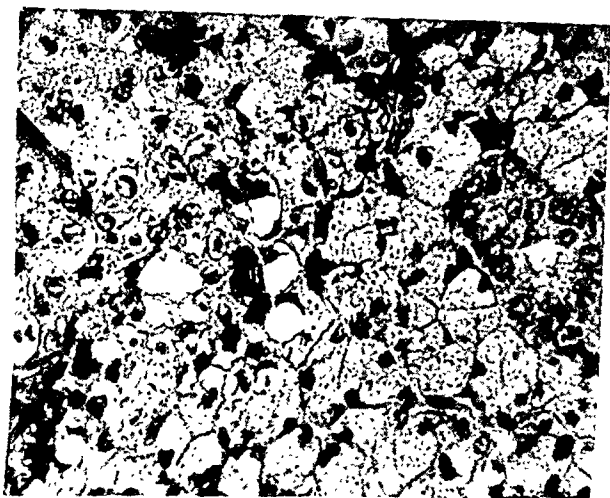


Fig. 1. Secretorically highly active cell complexes (in the centre of the picture) with strongly vacuolated cytoplasm and nuclei with fine-meshed chromatine network and prominent nucleolus surrounded by relatively inactive complexes with voluminous cytoplasm and strongly stainable nuclei. Submaxillary gland. Partial cauterization of the cervical sympathetic trunk. 400  $\times$ .

nuclei with prominent nucleolus. These cells exhibit all the characteristic signs of a strong stimulation, shown by cells possessing both intact sympathetic and parasympathetic innervation. The other type of acini has the same relatively intact appearance as those with sympathetic denervation described above. The terminal portions of the one and same type are generally gathered into smaller or bigger groups, sometimes filling out even a whole lobule. It is worth noticing that the secretorically relatively inactive acini are often immediately adjacent to highly active cell complexes (Fig. 1).

In principle the same results may be obtained after the partial destruction of the submaxillary ganglion and after partial cauterization of the nerves round the submaxillary artery (Fig. 2). In these cases, however, the effect caused by the injury becomes

practically identical with that obtained upon a combined sympathetic-parasympathetic denervation, as it is impossible — even with the most careful technique — to localize the injury on the isolated nerve fibres of either the sympathetic or the parasympathetic system. The secretorically inactive, or relatively inactive acini occurring in these cases generally belong to the sympathetic-ally, or sympathetically-parasympathetically denervated type.

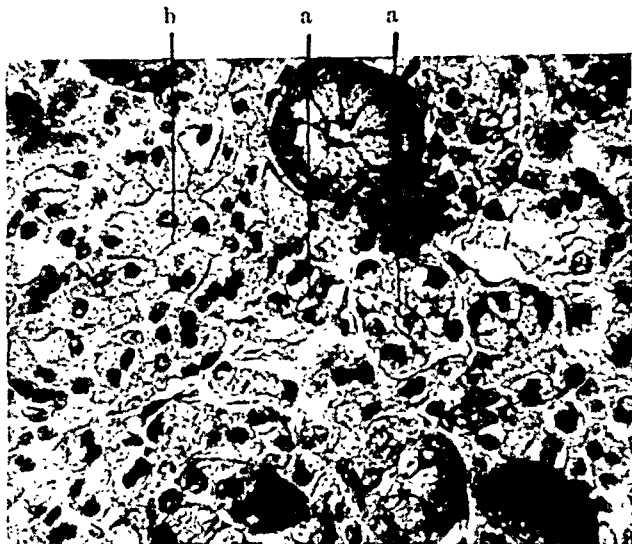


Fig. 2. Highly active cell complexes (a) adjacent to relatively inactive acini (b). Submaxillary gland. Partial cauterization of the submaxillary ganglion. 400  $\times$ .

Finally it should be emphasized that within the same terminal portion all the glandular cells — as far as cytologically can be judged — are always in the very same secretory stage.

*Thus after indirect stimulation both secretorically inactive or relatively inactive and secretorically highly active cell complexes are to be found in the partially denervated submaxillary gland, side by side, mixed with and adjacently joining to one another.*

## 2. Sublingual gland.

Following the applied stimulation the serous part in the sublingual gland shows no clear cytological signs of secretory activity. Thus the observations must be restricted to the quantitatively dominating mucous cells.

Following chordectomy the mucous cells are voluminous and filled with secretion. Even a long stimulation fails to change this picture. On the other hand the terminal portions possessing

intact parasympathetic innervation lose nearly all of their mucous character on stimulation: they become small, shrunken, and do not contain any basophil secretion.

In those experiments, where the submaxillary ganglia were partially cauterized, among the highly activated acini some tubules could be observed, the cells of which were completely filled up with secretion, without showing any secretory signs. These cell complexes might easily be interpreted as parasympathetically denervated ones. — Thus from a principal point of view this experiment gives the same results as those on the submaxillary gland reported above.

### Discussion.

On a long and intensive nervous stimulation of the salivary glands investigated marked cytological changes arise, signifying a high secretory activity. The gland shows a uniform histological appearance, which indicates that all the secretory portions are activated. After partial denervation the same stimulation results in quite another histological picture. This picture is dominated by two different types of acini. Cytologically judged these cell complexes must be interpreted as secretorically inactive or relatively inactive and secretorically highly active ones. It is obvious that the occurrence of the acini mentioned first is a consequence of denervation, but their development may of course be interpreted in two various ways differing completely in point of principle.

With regard to the previous experiments on the innervation of the adrenal medulla, it should be very reasonable to interpret the present observations as follows:

The salivary glands — like other autonomic effector systems — are innervated by a nervous ground plexus (HILLARP, 1946). This plexus innervates directly all the terminal portions. All the terminal portions are brought into secretory activity via this plexus on a stimulation that activates all the nerve cells in the salivary center. However every postganglionic neurone innervates only a limited number of acini in its course in the ground plexus. Thus this system is organized on the base of neuro-effector units. Therefore when applying the same stimulation after a partial transsection of the praeganglionic or postganglionic nerve fibres, only the secretory units — possessing still intact innervation — are activated, whereas the denervated cell complexes remain in-

active. — This interpretation explains totally all the results of the present investigation.

There exists in fact a possibility, however, of explaining the present observations even from the point of view of the CANNON-ROSENBLUETH diffusion theory. A transmitter substance which is liberated in or at certain cells and then diffuses to other neighbouring cells, would primarily activate the first-mentioned ones, and in the second place — and probably not to the same extent — the latter cells. In this way a picture of highly activated complexes alternating with more or less submaximally stimulated ones might develop.

However the plausibility of this latter interpretation is very questionable — as well with regard to the salivary glands, as concerning the adrenal medulla. The presence of acini without any cytological signs of secretory activity alternating with highly active cell complexes, immediately adjacent to one another, speaks against the assumption that a diffusion of a transmitter would be an important innervation mechanism. *In spite of their strategically poor position adjacent to highly active complexes and in spite of the sensibilization, which follows postganglionic denervation, these acini appear to be protected from any influence exerted by the chemical mediator.* This evidence with its possible objections has been thoroughly discussed in a previous publication (HILLARP, 1946). It is obvious that a leakage of the transmitter substance from the place of formation to the blood circulation — especially in the adrenergic system — may be present. From this fact, however, can by no means be concluded that this leakage does play an important rôle in the innervation of the effector system.

Already this consideration may convince us that it is very difficult to accept the theory advanced by CANNON and ROSENBLUETH concerning the functional organization of the peripheral autonomic nervous system. In addition it must be mentioned that the first postulate of their conception — *i. e.* that only a limited number of effector cells are directly innervated — is not now supported by any morphological evidence. This circumstance may be clearly seen from several new neurohistological investigations (See for instance BOEKE, 1940, HILLARP, 1946).

As we did not succeed in confirming the existence of submaximally stimulated cell complexes in the partially denervated salivary glands, it is not possible to demonstrate — in contrast

to the adrenal medulla — a convergency of different postganglionic neurons to the very same neuro-secretory unit.<sup>1</sup> *However our experiments strongly speak in favour of a functional organization of the peripheral autonomic nervous system based on the neuro-effector unit.* Thus in this respect the adrenal medulla does not seem to occupy any special position within the autonomic effector systems. It would be, however, too hasty inference to generalize the neuro-effector unit to hold for all of the other systems. It is conceivable that a diffusion of a transmitter substance within certain effector systems may play a more or less important rôle, and thus making the presence of these units illusive. This statement may be valid before all for the smooth musculature, where even in certain cases a syncytial arrangement of cells (See for instance BOZLER, 1938) may be assumed completely to modify the organization of the autonomic innervation.

MANSFELD, HECHT and KOVÁCS (1931) have previously — after partial denervation — observed the occurrence of secretorically active and inactive acini in the salivary glands on indirect stimulation. As their work otherwise has no bearing on the problems of the present paper, its further discussion will be omitted.

### Summary.

The reaction of the glandular cells in the submaxillary and sublingual glands of the rat has been cytologically investigated on indirect stimulation, before and after partial sympathetic or parasympathetic denervation. A clear explanation of the results obtained could be presented on the basis of neuro-secretory units in these glands. On the other hand a diffusion of the chemical mediators according to the view of CANNON and ROSENBLUETH seems not to have any significant importance for the innervation. Thus the present results support the conception concerning the functional organization of the peripheral autonomic nervous system advanced in a previous paper.

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<sup>1</sup> The presence of submaximally stimulated acini in the partially denervated submaxillary gland is conceivable on the base of the histological appearance, but the cytological changes are not clear enough to ascertain this assumption.

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## Vasomotor Reactions in Valsalva's Experiment.

An Electrocardiographic Study with Reference to the Effect of Smoking.

By

L. AUTIO, O. ERÄNKÖ and EEVA JALAVISTO.

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The changes in intrathoracic pressure occurring during and after the Valsalva experiment have strong circulatory effects. The idea of using the Valsalva strain as a circulatory fitness test is therefore appealing. All attempts have, however, failed, because grave alterations *e. g.* of impulse conduction in the heart, may appear even in perfectly healthy individuals. Furthermore, the reactions seem to have no bearing on any definite diseased condition of the heart. It has also been claimed that the experiment may not be quite harmless. It has been assumed that sudden death *e. g.* in swimming may be caused by an unfavorable reaction against increased intrathoracic pressure (PETERSEN, 1928). Only in estimating the fitness for special tasks, the experiment may be of value. *E. g.* pilots who will often be exposed to great gravitational forces, are regularly tested with Valsalva's experiment, partly in order to sight out those prone to vasomotor collapses, partly because increased intraabdominal pressure is a help against the "blacking out" caused by great accelerations and a pilot must be known to endure such increased pressures.

Haemodynamically Valsalva's experiment is characterized by the following events. During the strain the cardiac output, the pulse pressure and the size of the heart diminish through a deficient filling of the auricles. The mean arterial pressure usually rises. The initial rise is soon followed by a fall, which is later

replaced by a secondary rise in the pressure. The peripheral venous pressure is elevated as well (*e. g.* LIEDHOLM, 1939). The heart rate is usually accelerated and there appear some electrocardiographic changes, which are interpreted as showing an increased sympathetic influence *i. e.* increase in the height of the P wave and decrease in that of the T wave. After cessation of the strain an immediate drop in the mean and the pulse pressures takes place. After a latency of about 4 seconds this drop is followed by an increase in mean pressure and a high rise in pulse pressure (HAMILTON, WOODBURY and HARPER, 1936). In this phase the heart rate is very much slowed and disturbances of impulse conduction in the heart are seen. The latter changes are interpreted as signs of increased vagal activity. The changes during the straining are attributed to reflexes originating in extrathoracic arteries *i. e.* in the carotid sinus, whereas the slowing of the heart rate after the strain is due to stimulation of the aortic receptors as well (HAMILTON and al.).

We may leave out of discussion the receptory mechanism activated in the different phases of the test and only state that *the Valsalva experiment represents a type of strain in which an increased sympathetic activity is followed by a strong vagal one.* It may therefore be anticipated, that conditions interfering with the autonomic balance may modify the responses in the Valsalva experiment. Thus although the experiment cannot be used as a fitness test in general, it may be of value when the sympathetic and vagal reactivity are checked in a given subject.

Smoking is considered an agency interfering with the tone of the autonomic nervous system. The opinions as to the mode of action diverge, however, considerably. It was therefore deemed interesting to see whether the smoking could in some way affect the reactions in the Valsalva experiment. The differences were expected to be seen in the electrocardiograms, because similar ecg-changes which are encountered in the Valsalva experiment are reported to occur after smoking as well (alterations in the height of the P and T waves, the length of the P—Q time, frequency etc.).

### Methods and Material.

The experiments were carried out as follows. The experimental subject rested lying supine for 10 minutes before the beginning of the experiment. After a deep inspiration the subject strained against a

manometer trying to keep the pressure on 40—50 mm Hg for 30 seconds. The straining was begun with inflated lungs. The electrocardiograms were recorded with a "Triplex" electrocardiograph, which allows simultaneous recording of the three usual extremity leads. The film was exposed: 1) in rest before the experiment, 2) 15—20 seconds after the beginning of the straining, 3) 25 seconds after the beginning. In this phase a continual record was made which covered the end of the straining and the first 10—15 seconds after the beginning of breathing. After the first experiment the subject smoked 1—3 "Twenty Gold" cigarettes (Virginian tobacco). He was instructed to inhale the smoke. The smoking took 10—15 minutes according to the number of cigarettes smoked. Only a few experiments with three cigarettes were, however, made because most subjects became nauseated by this dose. The majority of the experiments were made in the morning — if done during the day it was made sure that the subject had not smoked for at least two hours preceding the experiment. In this time the effect of previous smoking was considered to have faded.

As controls two similar successive Valsalva experiments were carried out, but without smoking between the strainings.

The experiments were carried out on 25 apparently healthy medical students and members of the staff of the Institute. On thirteen out of these, the control experiments were made as well. 20 were males, 5 females, 13 habitual smokers, 12 non-smokers. Two cases had to be discarded because of possible pathological conditions (common cold, greatly increased P—Q time (0.26—0.29 sec.)).

In the statistical treatment of the results because of abnormal distribution of the values as a rule only methods of homograde statistics were used instead of the more cumbersome methods of heterograde statistics. Thus *e. g.* the  $\chi^2$ -method was used throughout when possible, *i. e.* when no class was represented by a value less than .5. In most cases the class correlation coefficient was calculated according to the following scheme, in which the letters a—i represent number of cases falling into each class. The correlation coefficient gets in this case the form:

$$r^2 = \frac{(-a + c + g - i)^2}{(a + c + d + f + g + i)(a + b + c + g + h + i)}$$

(KUKKAMÄKI). The mean error of  $r$  is calculated according to the equation

$$\frac{1 - r^2}{\sqrt{n - 1}}.$$

$\begin{array}{c} x \\ y \end{array}$	-1	0	+1
-1	a	b	c
0	d	e	f
+1	g	h	i

Table 1.  
Non-smokers.

Date. Exp. Subject.	Initial resting Heart Rate	Changes caused by smoking in			
		Resting Rate beats/ min.	P wave height mm.	P—Q time sec.	T wave height mm.
1 cigarette:					
2. 11. 46 L. A. ....	80	+ 13	± 0	— 0.03	— 0.1
22. 3. 47 L. A. ....	79	— 8	— 0.3	— 0.01	— 0.3
19. 12. 46 Pa. ....	69	— 7	± 0	— 0.01	± 0
7. 10. 47 Ha. ....	56	+ 5	± 0	+ 0.02	— 0.5
29. 10. 47 Ec. ....	47	+ 10	— 0.8	— 0.01	— 1.0
1. 11. 47 Sa. ♀ ....	66	+ 19	± 0	± 0	— 0.1
30. 11. 47 Ta. ....	61	— 1	± 0	— 0.01	+ 0.3
25. 10. 47 Lou. ....	95	— 5	± 0	— 0.01	± 0
30. 10. 47 H—d. ....	88	+ 2	+ 0.3	± 0	± 0
15. 11. 47 He. ....	95	— 15	+ 0.1	+ 0.01	+ 0.2
23. 11. 46 E. J. ♀ ....	64	— 3	± 0	± 0	± 0
2 cigarettes:					
22. 3. 47 L. A. ....	79	— 2	— 0.3	± 0	+ 0.3
19. 2. 47 P—a. ....	69	— 2	— 0.5	— 0.01	± 0
23. 11. 47 E. J. ♀ ....	64	— 6	± 0	± 0	± 0
3 cigarettes:					
7. 5. 47 Ve. ....	64	— 4	± 0	+ 0.01	± 0
8. 5. 47 Ta. ....	63	— 2	— 0.1	± 0	± 0
4. 10. 47 Ha. ....	64	— 9	± 0	± 0	— 0.8
17. 6. 47 L. A. ....	65	+ 3	+ 0.2	+ 0.01	+ 0.2

## Results.

### The Effect of Smoking on the Ecg. in Rest.

*Heart Rate.* The resting pulse rate varied in different subjects, being 56—95 beats/min. in non-smokers and 51—81 in smokers. The averages for both groups were 70 and 68 respectively. After smoking the resting heart rate was differently affected in the two groups. Whereas the non-smokers group did not show any difference as compared to the pre-smoking values, the group of smokers showed a regular rise in the frequency amounting to 39 beats/min. In one single subject after smoking of three cigarettes a drop in the frequency of 8 beats/min. was noted.

Experiments carried out on different days sometimes gave divergent results. It may, however, be noted that in non-smokers the control and the main experiments gave regularly the same result whereas in the smokers group in every case but one the smoking and control resting heart rates differed, just as would

Table 2.

*Smokers.*

Date. Exp. Subject.	Initial resting Rate	Changes caused by smoking in			
		Resting Rate	P wave height mm.	P—Q time sec.	T wave height mm.
1 cigarette:					
7. 11. 46 O. E. ....	59	+ 26	+ 0.1		— 0.5
2. 4. 47 O. E. ....	66	+ 1	— 0.3	+ 0.01	± 0
23. 11. 46 Mu. ♀ ....	69	+ 37	± 0	± 0	— 0.9
23. 11. 46 E. M. ....	59	+ 14	+ 0.3	± 0	— 0.8
5. 11. 47 Vi. ....	64	+ 22	± 0	— 0.01	± 0
31. 10. 47 Lu. ....	51	+ 23	± 0	+ 0.01	± 0
» » » Pii. ....	74	+ 19	+ 0.6	± 0	— 0.3
27. 10. 47 Pa. ....	55	+ 14			
3. 11. 47 Sā. ♀ ....	71	+ 25	+ 0.6	± 0	— 1.4
30. 11. 47 Tw. ....	81	+ 5	— 0.3	+ 0.01	± 0
2 cigarettes:					
7. 11. 46 O. E. ....	59	+ 19	± 0		— 0.7
23. 11. 47 Mu. ♀ ....	69	+ 39	± 0	± 0	— 1.1
3 cigarettes:					
29. 4. 47 Hā. ....	54	+ 10	+ 0.3	± 0	± 0
5. 5. 47 Ki. ....	69	+ 7	— 0.3	+ 0.01	— 1.5
26. 9. 47 Te. ....	61	+ 5	± 0	± 0	± 0
6. 5. 47 Tw. ....	64	— 8	± 0	+ 0.01	+ 0.3

be expected if the effect of smoking on the heart rate were a real one. In tables 1—3 the columns 2 give the initial resting heart rate, the column 3 the difference between the resting post-smoking frequency and the initial value. Table 1 shows the values of the nonsmokers, table 2 those of the smokers. In table 3 the values for the control experiments are compiled. In table 3 column 4 the difference of the values in control and smoking experiments in the same subject are given. It confirms the impression gained when the tables 1 and 2 are compared.

Table 4 summarizes the results obtained regarding the effect of smoking on the heart rate. It shows the distribution of increased and decreased heart rates in the three experimental groups. The distribution is the same in the control- and non-smokers group which therefore can be treated as one. The distribution corresponds to a correlation coefficient  $+ 0.68 \pm 0.09$  which is statistically significant showing that the effect of smoking is different in smokers and non-smokers. (Calculated from the table with class limits of  $\pm 10$  beats/min.)

Table 3.  
Control experiments.

Date	Subject		Initial Heart Rate (I)	Diff. between initial Rates I and II	Diff. between initial post smoking and Rate II	Alterations induced by the first experiment in		
						P wave height	P—Q time	T wave height
7. 11. 46	O. E.	smoker	59	+ 6	+ 20	± 0		± 0
23. 11. 46	E. M.	»	59	— 4	+ 18	± 0	± 0	— 0.3
» » »	»	»	167	— 7	+ 21	± 0	± 0	+ 0.3
22. 11. 47	Vi.	»	72	+ 2	+ 20	+ 0.3	+ 0.01	± 0
» » »	Pii.	»	75	— 5	+ 24	± 0	± 0	+ 0.6
» » »	Pa.	»	67	+ 11	+ 3	+ 0.1	— 0.01	± 0
» » »	Sä. ♀	»	75		+ 32	— 0.1	— 0.01	— 0.2
» » »	Vu.	»	83	— 8	+ 13	— 0.4	± 0	— 0.1
» » »	Ee.	non-smoker	49	+ 5	+ 5	± 0	± 0	— 0.2
23. 11. 46	E. J. ♀	»	55	+ 9	— 5	± 0	± 0	± 0
» » »	»	»	162	— 1	+ 4	— 0.1	— 0.01	± 0
» » »	»	»	263	— 5	± 0	± 0	+ 0.01	+ 0.1
22. 11. 47	Lou.	»	80	— 5	± 0	± 0	— 0.01	± 0
» » »	H—d	»	80	— 15	+ 17	— 0.5	— 0.01	— 0.1
» » »	He.	»	78	— 7	— 8	+ 0.2	± 0	± 0

Table 4.

Effect of smoking on resting heart rate. In parenthesis values of single experiments, including more than one value obtained with the same subject.

	Change in Heart Rate after smoking beats/min.							
	< 0	0 + 10	> + 10	Σ	< — 5	— 5 + 5	> + 5	Σ
Controls ....	8 (10)	4 (4)	1 (1)	13 (15)	5 (5)	6 (8)	2 (2)	13 (15)
Non-smokers.	7 (12)	3 (4)	1 (2)	11 (18)	1 (5)	8 (10)	2 (3)	11 (18)
Smokers ....	1 (1)	3 (5)	8 (10)	12 (16)	0 (1)	2 (3)	10 (12)	12 (16)
	16 (23)	10 (13)	10 (13)	36 (49)	6 (11)	16 (21)	14 (17)	36 (49)

In the tables 1—3 some other electrocardiographic findings are presented as well. The three last columns give the differences of the pre- and post-smoking resting values of P—Q-time in sec., P amplitude and T amplitude in mm. (= 0.1 mV.). As seen from the table the smoking has very little effect on these quantities. *E. g.* variations in the height of P exceeding 0.5 mm. occur only 5 times and one of these changes is seen in the control experiments.

<sup>1</sup> Both experiments carried out after smoking of one cigarette.

<sup>2</sup> After smoking of two cigarettes.

Table 5.

	Change in the height of							
	P-wave				T-wave			
	after smoking							
	$\leq -0.3$	$-0.2$ $+0.2$	$\geq +0.3$	$\Sigma$	$\leq -0.3$	$-0.2$ $+0.2$	$\geq +0.3$	$\Sigma$
Controls ....	1	10	2	13	1	10	2	13
Non-smokers.	2	8	1	11	3	8	0	11
Smokers ....	1	6	4	11	6	5	0	11
	4	24	7	35	10	23	2	35

When present the direction of the change is however in conformity with what would be expected on ground of the effect of smoking on the frequency — in smokers the changes are in the direction of increased sympathetic tone in the control experiments and in non-smokers the height of P is decreased as in increased vagal tone. The P—Q time does not change. As often mentioned the T waves are sometimes altered after smoking. The effect is not a constant one but when present it indicates a decreased height of the T wave. This is only natural, since according to ASHMAN and collab. (1945) increase in the heart rate usually leads to a depressed height of the T wave.

In table 5 the changes observed in the amplitude of the P and T waves are summarized. The distribution of differences amounting to or exceeding 0.3 mm. in the three experimental groups are shown in the table.

#### Valsalva's Experiment.

a) *Heart Rate.* Two different types of responses are seen in Fig. 1 in which the heart rate is plotted against time after the beginning of straining. The broken lines represent experiments after smoking in the subjects Ve and Ha ( $A_1$ ,  $B_2$  and  $B_3$ ). Both subjects were non-smokers and the difference in initial heart rate was negligible. As seen there is no essential difference between the reactions after smoking and before it; the interindividual differences are on the other hand quite remarkable although some variations may occur on different days in the same subject, as also seen in fig. 1. The curves  $B_1$  and  $B_2$  are obtained 3 days

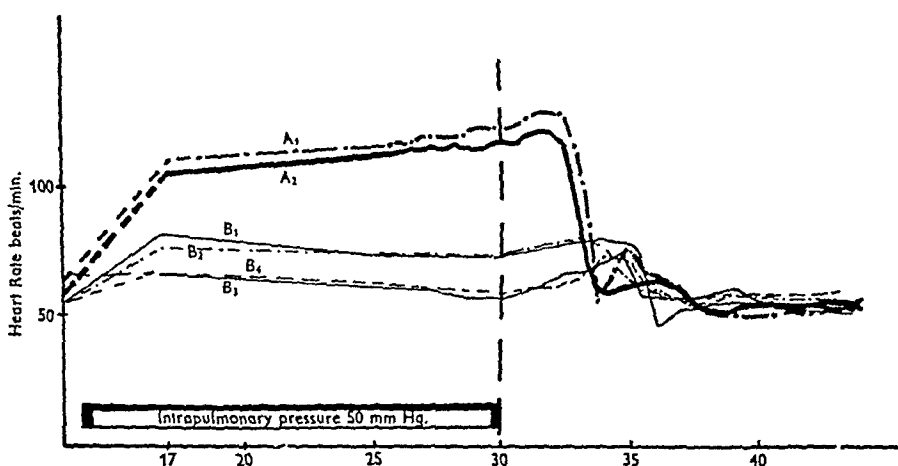


Fig. 1. Heart rate response in Valsalva's experiment before and after smoking. Dotted lines after smoking. 7. 5. 47. Ve.  $A_1$  after 3 cigarettes.  $B_1$  and  $B_2$  7.10.47. Ha. 1 cigarette.  $B_3$  and  $B_4$  4. 10. 47 Ha. 3 cigarettes.

after the experiments represented by  $B_3$  and  $B_4$ . The resting pulse rate does not differ and cannot explain the differences in reactivity. The biggest difference between the A and B curves occurs at the end of the straining. The post-straining phase is on the contrary very similar in different individuals. It is in general especially characterized by a sudden drop in the frequency to a low level, which is fairly constant even interindividually. In 14 cases out of 19 this level is 50—60 beats, in one case lower, and in 4 higher, 65—80. The low level value is not dependent on the initial resting heart rate. In 4 subjects there is no typical sudden drop and the frequency gradually sinks to the initial level (Fig. 2c).

As mentioned the smoking has not affected the initial heart rate in the curves of fig. 1. It is therefore reasonable to question, whether the size of the response would be altered, if the post-smoking resting value would differ from the pre-smoking value. Very often the rise in the heart rate is quite parallel both in the pre- and post-smoking curves (Fig. 2 a, b, c) i. e. the differences of post- and pre-smoking values remain the same during the straining. Sometimes, however, the smoking seems to have affected the reactivity in a direction opposite to the effect of smoking on the resting heart rate. (Fig. 2 d, e.) Evidently the size and shape of the response cannot be anticipated even when the effect of smoking on the resting value is known. In order to get some measure for differences in reactivity the following method has been adopted. The frequencies in each experiment have been plotted against time and the area of the resulting curve above and



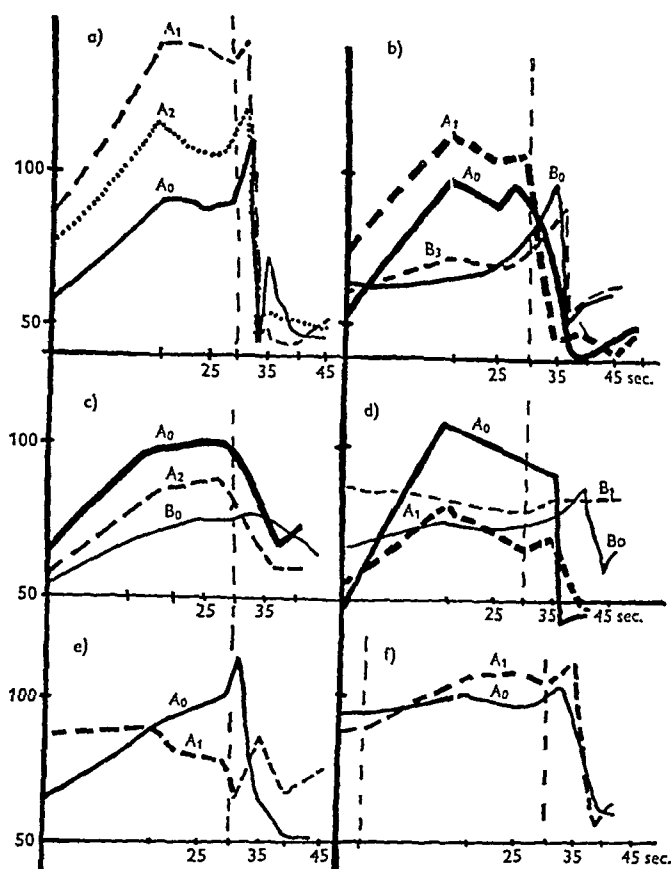


Fig. 2. a) O. E. 7. 11. 46. Dotted lines after smoking. 1 and 2 cigarettes. b)  $A_0$  and  $A_1$  31. 10. 47 Lu. 1 cigarette.  $B_0$  and  $B_1$  8. 5. 47. Ta. 3 cigarettes. c)  $A_0$  and  $A_1$  23. 11. 46. E. J. 2 cigarettes.  $B_0$  29. 4. 47. Hä. d)  $A_0$  and  $A_1$  29. 10. 47. Ee. 1 cigarette.  $B_0$  and  $B_1$  1. 11. 47. Sa. 1 cigarette. e) 5. 11. 47. Vi. 1 cigarette. f) 25. 10. 47. Lou. 1 cigarette.

below the resting heart rate level has been measured planimetrically. In this way we get in arbitrary units (sq. mm.  $\times$  10)

$$[10 \text{ sq. mm.} = \frac{10 \text{ beats/min.} \times 5}{4.9} = \approx 1/6 \text{ beats}] \text{ the total in-}$$

crease and total decrease in heart rate during 45 seconds, (however, with the exception of the increase in the first 10—15 seconds after the beginning of straining, which was not recorded). The heart rate responses recorded in this way during the Valsalva's experiment range from 8—162. In 8 subjects the reactions have been recorded twice under similar conditions. The reactions differed in 5 cases less than 10 units, the average being 17 units. If two reactions differing for 20 or more units are judged as dis-

Table 6.

Reaction Exp. subj.	decreased for $\geq 20$ units	No change	increased for $\geq 20$ units	$\Sigma$
Nonsmokers .....	5	3	3	11
Smokers .....	7	4	1	12
	12	7	4	23

Table 7.

Reaction in units Experiment	$\geq 50$	$< 50$	$\Sigma$	$\geq 65$	$< 65$	$\Sigma$
Before smoking .....	5	18	23	7	16	23
After " .....	11	12	23	13	10	23
	16	30	46	20	26	46

similar and the distribution of increased and decreased reactions are enumerated we get the table 6.

As seen from table 6 the non-smokers and smokers groups do not differ from each other. On the whole the decreased reactions after smoking seem to be more common than an enhanced reaction (12 cases against 4). If we compare the size of the reaction before and after smoking in dividing the material into two groups with great and with small reactions, a similar result is obtained. In table 7 two different classifications are represented. The distribution of post- and pre-smoking values is not much affected by a change in the class limits and shows that smaller reactions are somewhat more common among the post-smoking values. The difference is not statistically ascertained,  $P$  being about 5 % ( $\chi^2 = 3.4$  and  $3.2$ ) but it shows that the difference may possibly be a real one.

The effect might furthermore be dependent upon the amount of cigarettes smoked. The decreasing effect of smoking is thus recorded with striking evidence in a subject (Ki.) after smoking of 3 cigarettes. Two experiments were carried out before smoking with exactly the same response (70 units), but after smoking there was no response at all ( $-26$  units). Fig. 3 is constructed as fig. 1 and shows clearly the striking difference in reactivity before and after smoking. The subject was nauseated, which is of course already a sign of autonomic disturbance.

Evidently smoking does not at least increase the reactivity in Valsalva's experiment.

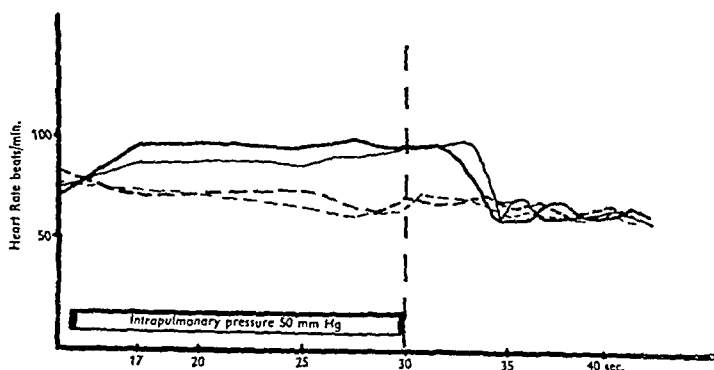


Fig. 3. 5.5.47. Ki. Heart rate response in Valsalva's experiment. Dotted lines after smoking of 3 cigarettes.

b) *P—Q time. Height of the P and T Waves.*

When considering the big differences in reactivity, which occur spontaneously or may be induced by *e. g.* smoking, the question arises whether any electrocardiographic signs may be found, which correlate with the reactivity of the subject. As well known the height of the P wave is considered to be dependent upon the action of the heart nerves, but a comparison is only possible between different records of the same subject and not between different individuals, the voltage of the deflections being of course, much affected by secondary conditions *e. g.* electrical conductivity of the skin, direction of electrical axis of the heart etc. The same holds good for the amplitude of the T deflection. On the other hand differences in autonomic balance alter the duration of the P—Q interval in a given individual. *E. g.* increase in vagal tone tends to lengthen the P—Q time, increased sympathetic activity leading to the reverse alteration. It is therefore perhaps worth while to compare the values of P—Q to the reactivity in the Valsalva experiment in different individuals, especially as the P—Q time is not much affected by different recording conditions. On the other hand the P—Q time does not vary much even in different individuals. It must furthermore be borne in mind that the length of P—Q time in a given subject might be long or short owing to *e. g.* anatomical differences, which have, of course, no bearing on the autonomic balance. An indication to a possible correlation was found, when the P—Q durations belonging to the curves in Fig. 1 were compared. The subject Ve. (curves A) with high reactivity had a very short P—Q interval (0.13), the one with lacking or small reactions Ha. (curves B)

Table 8.  
Non-smokers.

Date	Subj.	Ciga- rettes	Resting pulse rate	P—Q rest	P—Q strain	Reac- tion (arb. units) <sup>1</sup>	Mean electrical axis			
							rest	strain	Dif- ference strain— rest	Dif- ference pre-post- smoking
2. 11. 46	L.A.	0	80	0.16	0.12	81/17	+97°	+104°	+ 7°	
— " —	"	1	93	0.13	0.12	99/30	+97°	+120°	+ 23°	± 0°
22. 3. 47	"	0	79	0.14	0.13	83/14	+98°	+113°	+ 15°	
— " —	"	1	71	0.13	0.12	103/6	+94°	+117°	+ 23°	— 4°
— " —	"	2	77	0.14	0.13	67/3	+95°	+112°	+ 17°	+ 1°
19. 12. 46	P-a	0	69	0.16	0.14	85/5	+40°	+ 76°	+ 36°	
— " —	"	1	62	0.16	0.14	127/6	+40°	+ 75°	+ 25°	+ 0°
— " —	"	2	67	0.17	0.13	134/8	+12°	+ 67°	+ 55°	— 28°
4. 10. 47	Ha.	0	64	0.20	0.18	0/0	+55°	+ 68°	+ 13°	
— " —	"	3	55	0.20	0.18	13/0	+58°	+ 64°	+ 6°	+ 3°
7. 10. 47	"	0	56	0.20	0.18	65/3	+54°	+ 70°	+ 21°	
— " —	"	1	61	0.22	0.20	37/3	+50°	+ 67°	+ 17°	— 4°
23. 11. 46	E. J. ♀	0	64	0.15	0.14	93/0	+79°	+ 88°	+ 9°	
— " —	"	1	61	0.16	0.14	17/0	+83°	+ 90°	+ 7°	+ 4°
— " —	"	0	55	0.15	0.12	93/0	+83°	+ 82°	— 1°	
— " —	"	2	58	0.16	0.14	75/0	+80°	+ 90°	+ 10°	— 3°
29. 10. 47	Ee.	0	47	0.16	0.13	146/1	+65°	+ 90°	+ 25°	
— " —	"	1	57	0.15	0.12	52/5	+63°	+ 87°	+ 24°	— 2°
1. 11. 47	Sä. ♀	0	66	0.16		31/4	+24°	+ 58°	+ 34°	
— " —	"	1	85	0.16	0.15	0/17	+21°	+ 56°	+ 35°	— 3°
8. 5. 47	Ta.	0	63	0.18	0.15	26/3	— 43°	— 98°	— 55°	
— " —	"	3	61	0.18	0.15	37/2	— 57°	— 101°	— 44°	— 14°
30. 11. 47	"	0	61	0.19	0.15		— 47°	± 0	+ 47°	
— " —	"	1	60	0.19	0.14		— 38°	— 92°	— 54°	+ 9°
25. 10. 47	Lou.	0	95	0.19	0.17	6/29	+99°	+ 99°	± 0	
— " —	"	1	90	0.18	0.17	43/20	+95°	+100°	+ 5°	— 4°
30. 10. 47	H-d	0	88	0.18	0.16	66/8	— 8°	— 40°	— 32°	
— " —	"	1	90	0.18	0.16	3/9	— 7°	— 35°	— 28°	+ 1°
15. 11. 47	He.	0	95	0.14	0.13	53/36	+57°	+ 75°	+ 17°	
— " —	"	1	80	0.14	0.14	110/28	+37°	+ 68°	+ 31°	— 20°
7. 5. 47	Ve.	0	64	0.13	0.12	112/10	+42°	+ 68°	+ 26°	
— " —	"	3	60	0.14	0.13	116/8	+42°	+ 69°	+ 27°	± 0°

a long P—Q time (0.20). In tables 8 and 9 columns 5 and 6 show the resting and straining P—Q intervals; in column 7 the size of the reaction is given in units explained above. The reactivity evidently shows no correlation either to the resting P—Q length or to the straining P—Q value. The difference in P—Q time corresponding to the different reactivity in the subjects A and B, fig. 1, is therefore probably only occasional.

It may be objected that the reactivity measured as presented above might not be an adequate measure of the reactions of the

<sup>1</sup> Left-hand figure = total increase in heart rate.  
Right-hand " = " decrease " " " .

Table 9.

Smokers.

Date	Subj.	Ciga- rettes	Resting heart rate	P—Q rest	P—Q strain	Reac- tion (arb. units)	Mean electrical axis			
							rest	strain	Dif- ference strain— rest	Dif- ference pre- post- smoking
7. 11. 46	O.E.	0	59	0.18	0.17	83/4	+47°	+ 57°	+ 10°	
— » —	»	1	85	0.18	0.15	121/46	+48°	+ 59°	+ 11°	+ 1°
— » —	»	2	78	0.18	0.15	80/28	+46°	+ 56°	+ 10°	— 1°
2. 4. 47	»	0	66	0.17	0.14	95/13	+47°	+ 42°	— 5°	
— » —	»	1	67	0.18	0.15	92/14	+46°	+ 41°	— 5°	— 1°
23. 11. 46	Mu.♀	0	69	0.13	0.12	70/0	+63°	+ 72°	+ 9°	
— » —	»	1	106	0.13	0.12	10/5	+63°	+ 69°	+ 6°	± 0°
— » —	»	2	108	0.13	0.12	44/34	+67°	+ 74°	+ 7°	+ 4°
— » —	E.M.	0	59	0.15	0.14	30/5	+82°	+ 94°	+ 12°	
— » —	»	1	73	0.15	0.14	31/14	+80°	+ 96°	+ 16°	— 2°
27. 10. 47	Pa.	0	55	0.18	0.14	122/2	+70°	+ 86°	+ 16°	
— » —	»	1	69		0.16	79/2		+ 78°		
31. 10. 47	Lu.	0	51	0.18	0.15	111/8	+90°	+ 97°	+ 7°	
— » —	»	1	74	0.19	0.16	76/29				
— » —	Pii.	0	74	0.14	0.14	63/8	+66°	+ 90°	+ 24°	
— » —	»	1	93	0.14	0.14	88/36	+68°	+ 88°	+ 20°	+ 2°
5. 11. 47	Wi.	0	64	0.18	0.17	72/11	+60°	+ 70°	+ 10°	
— » —	»	1	86	0.17	0.16	3/25	+53°	+ 66°	+ 13°	— 7°
3. 11. 47	Sä.♀	0	71	0.14	0.13	89/2	+ 8°	+ 67°	+ 59°	
— » —	»	1	96	0.14	0.12	59/27	+13°	+ 59°	+ 46°	+ 5°
30. 11. 47	Tw.	0	81	0.15			+76°	+ 95°	+ 19°	
— » —	»	1	86	0.16			+75°	+ 84°	+ 9°	+ 3°
6. 5. 47	»	0	64	0.14	0.14	156/21	+75°	+120°	+ 45°	
— » —	»	3	56	0.15	0.15	162/3	+70°	+ 97°	+ 27°	— 5°
29. 4. 47	Hä.	0	54	0.20	0.17	67/0	+57°	+ 62°	+ 5°	
— » —	»	3	64	0.20	0.18	38/5	+58°	+ 75°	+ 17°	+ 1°
29. 10. 47	»	0	58		0.16	71/14	+57°	+ 62°	+ 5°	
— » —	»	1	53	0.18	0.15	45/0				
5. 5. 47	Ki.	0	69	0.18	0.17	70/1	+38°	+ 90°	+ 52°	
— » —	»	3	76	0.21	0.20	0/26	+18°	+ 80°	+ 62°	— 20°
26. 9. 47	Te.	0	61	0.16	0.13	54/12	+86°	+ 90°	+ 40°	
— » —	»	3	66	0.16	0.13	72/20	+82°	+100°	+ 18°	— 4°

autonomic nervous system of the heart under the experimental conditions of Valsalva's experiment. It is, however, possible to show that a correlation exists between the number of reactivity units and the change in the height of P and T deflections caused by the strain. Tables 10 and 11 show the distribution of P wave increase and T wave decrease during straining as compared with the corresponding resting values in different classes of reactivity. As seen from the tables small reactivity corresponds to small alterations in the height of P and T waves and vice versa. The difference is at and above the 0.27 % probability level indicating statistical significance.

Table 10.

Reactivity units \ P increase mm.	0—0.7	0.8—1.5	1.5	$\Sigma$
< 50 .....	8	10	0	18
50—90 .....	10	11	2	23
> 90 .....	2	6	9	17
	20	27	11	58

Table 11.

Reactivity units \ T decrease in mm.	0—0.9	1.0—1.9	2.0	$\Sigma$
< 50 .....	7	8	3	18
50—90 .....	6	11	6	23
> 90 .....	2	5	9	16
	15	24	18	57

The correlation might be regarded as an indication of the fact that the units used are probably quite serviceable as a measure of reactivity.

c. *Mean electrical axis.*

According to LAMMERT and SCHLOMKA (1935) the amplitudes of R and S waves are altered during and after the Valsalva experiment. The authors claim that the reduction of the amplitude of R and increase in the amplitude of the S wave is particularly great in cases which react to the straining less favourably, showing more severe electrocardiographic disturbances *e. g.* in impulse conduction than others with less conspicuous alterations in the amplitude of R and S waves. Particularly at the end of the straining the height of the R wave changes much; the amplitude may be reduced even to half of its original value. Because earlier Valsalva eeg:s have usually been recorded with one single lead (lead II) the alterations in amplitude are difficult to interpret. They may simply be caused by a great change in the mean electrical axis of the heart. Since the behaviour of the electrical axis has as far as we know, not been given any consideration in connection with

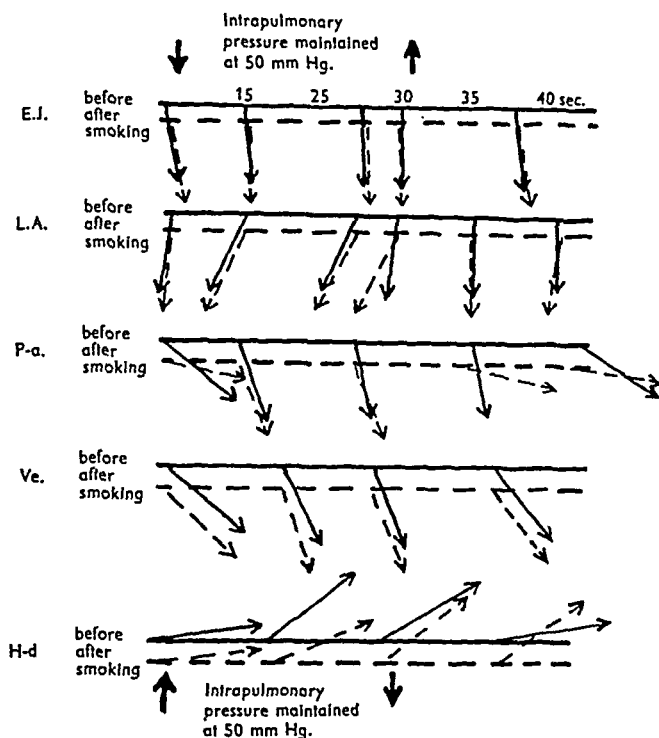


Fig. 4. Mean electrical axis of the heart in different phases of the Valsalva experiment. Dotted arrows after smoking.

the Valsalva experiment, the records have been analyzed in this respect. The mean axis has been calculated in the usual way with the help of the Dieuaide's chart. The direction of the mean electrical axis determined on different occasions in the same subject is fairly constant. In 14 out of 17 the paired determinations differed less than  $4^\circ$ , the average difference being  $1.9^\circ$ . The deviation caused by straining amounted to  $19-25^\circ$  on an average and is thus well above the limits of possible experimental errors. In fig. 4 some examples of reactions of the axis of hearts with differing resting mean electrical axis are shown. The arrows are arranged so as to show the direction of the mean electrical forces in different phases of the experiment, before, after 15 and 25—30 seconds of straining and again after the strain. Usually the straining causes a rightward deflection which passes off after the end of the straining. The axis has not been drawn in the figure more frequently, the values left out always being intermediate between those shown in the figure. No sudden changes in opposite directions *i. e.* pendulum movement of the mean axis was ever

Table 12.

Axis Shift°	Reactivity units				$\Sigma$
		< 50	50—90	> 90	
16	15° .....	9	9	6	24
	30° .....	4	7	8	19
	> 30° .....	5	6	3	14
		18	22	17	57

noted in the records. As seen from the figure, the rightward deflection is not always smaller in a heart with a nearly vertical axis than in one with a more horizontal resting mean axis. It seemed that the straining very rarely changed the direction leftwards; actually it happened at least twice, in the third case the leftward change was only 5 degrees which may be a random variation, although the change occurred consistently both in the pre- and post-smoking experiments. Both the other cases (Ta, H—d) with a change amounting to 30—50 degrees were cases with initial left axis deviations. It is questionable whether this can be considered as being quite normal. The subjects in question showed, however, no circulatory impairment or diseased heart (X-ray control). In tables 8 and 9 the initial direction of the electrical axis, the straining value, the difference due to strain and the difference between resting pre- and post-smoking values are given in columns 8—11. As is to be expected the smoking does not as a rule affect the direction of the axis. There are, nevertheless, 5 cases in which the electrical axis changed 7, 14, 20, 20, 28 degrees to the left. The shift is well beyond the experimental error at least in 4 cases, but its significance and the mechanism by which it is brought about are not clear.

The main interest in this connection is, of course, directed to the possible correlation between the reactivity of the subject measured as total increase of heart rate due to straining, and the change induced thereby in the direction of the electrical axis of the heart. Furthermore, the effect of strain in the pre- and post-smoking scores regarding the shift of the axis direction is to be compared. As to the latter, it can be stated that no difference seems to exist. Neither do smokers and non-smokers differ in the magnitude of the axis deviation caused by the strain. The



slight difference actually seen seems to depend upon the fact that the mean electrical axis of the non-smokers is on an average a little more to the left than that of the smokers (non-smokers  $+46^\circ$ , smokers  $+62^\circ$ ). In table 12 the possible correlation between axis shift and reactivity of the subject are compared. No correlation could be seen, although many class limits were checked. Probably the direction of the electrical axis during the strain is affected more by the anatomically altered position of the heart than by any intracardiac change.

### Discussion.

Although the material presented above is not extensive, some indications regarding the vasomotor effects of smoking may deserve consideration. We cannot, of course, decide which component in the complex act of smoking has been effective, the CO inhaled, the nicotine contained in the tobacco or organic distillation products etc. We state, at all events, that some effect exists and that it acts differently in smokers and non-smokers, since the heart rate seems to be more often increased in the smokers than in the non-smokers. If this difference is a real one, the question arises, what may be the explanation. There are at least three possibilities, so far as we can see. We may call the effect of smoking when it increases the heart rate a stimulating one. One could perhaps assume that only those subjects on whom smoking has a stimulating effect become smokers, others having, so to speak, no reason to smoke. On the other hand it may be a question of dosage and an adaptation to smoking. Thus it is quite possible that the effect of smoking is always depressing if only the amount of tobacco smoked is big enough. The depressing effect would be preceded by a stimulating one. In non-smokers the depressing dose is easily attained, whereas in smokers it is reached first after very heavy smoking. The few experiments with three cigarettes point in this direction. The third assumption would be that smoking has affected the reactivity of the autonomous nervous and endocrine system in increasing the susceptibility of the smoker to the effect of smoking. The experiments presented above do not give any evidence for such a conclusion nor do they contradict it, but the fact that no difference in reactivity against the Valsalva's strain has been found between smokers and non-smokers makes the supposition somewhat unlikely. It can, of course, be

objected that the way of measuring the reactivity which has been used may not be an adequate method. It is nevertheless some criterion — *i. e.* more than no evidence at all. The different action of smoking on smokers and non-smokers has been recorded at least once before. KOEHLER, HILL and MARSH (1947) when dealing with the effect of smoking on digestion stated that the bile secretion was diminished to a greater extent in smokers than in non-smokers by smoking of three cigarettes.

There is no reason to speculate here on the effect of smoking on the electrocardiogram. The changes are not great or consistent and the papers dealing with it controversial. (See *e. g.* LEFESCHKIN, 1942). It is probably much a question of individual susceptibility and of doses which determines whether and what sort of changes are obtained.

The reactivity as measured by the response of the heart rate to a strain in which the intrapulmonary pressure is maintained at 40—50 mmHg for 30 seconds, varies in different individuals and even on different occasions in the same subject. Sometimes a change in reactivity may be brought about with heavy smoking resulting in nausea. The initial resting heart rate need not be lowered in that case and the lack of reaction during straining can hardly be explained simply as caused by increased vagal tone. There is as a rule no relation between size of the reaction and the initial pulse rate. This makes it improbable that changes in reactivity could be caused by increased or decreased level of sympathetic or vagal activity. It is in accordance with the conception of autonomic nervous balance: the sympathetic and vagus are tonically active; an increase or decrease in the activity of one results in a compensatory reaction in the other (*e. g.* GELLHORN, 1943).

The changes in reactivity can therefore only be explained by assuming either that the actual stimulus affecting the cardio-accelerator mechanism varies or that there are differences in the susceptibility of the heart to a given stimulus. It is difficult to conceive, why the actual stimulus should be altered in straining by *e. g.* smoking, as the intrapulmonary pressure is maintained on the same level throughout the straining, the electrical axis shows the same deviation etc. Of course, it would be possible to postulate that the changes in *blood pressure* are the actual stimuli increasing or decreasing the pulse rate through the pressoreceptor mechanism of the carotid sinus or the aortic depressor nerve, but then again it must be asked why the pressure does not rise

or decline in the same way on different occasions. *Evidently it is necessary to assume an altered extra- or intracardiac susceptibility against the changed haemodynamic conditions.*

The eventual correlation of the reactivity as measured by the total increase in heart rate in Valsalva's experiment with unfavourable or favourable vasomotor conditions cannot be anticipated on basis of the present work. In several papers the Valsalva experiment is recommended, and actually used, as a test *e. g.* in checking the fitness of pilots, but a simple and practical method of estimating the vasomotor phenomena, *e. g.* the changes in blood pressure during the strain, is still lacking. Values obtained with the Riva-Rocci-Korotkow method may be misleading as they do not entirely agree with those obtained with the Hamilton manometer. The latter again cannot be handled as a routine method (RUSHMER, 1947). In view of these difficulties it might perhaps be of some value to estimate the reactivity on basis of the heart rate changes, using instead of the electrocardiographic records direct pulse counters *e. g.* that published by ZOTTERMAN and LUNDGREN (1945). The pulse curve may then be measured planimetrically in order to obtain a quasi quantitative measure of reactivity of the subject.

Electrocardiographically the most interesting phase of the Valsalva experiment is that after the end of the strain. There occur many signs of peculiar disturbances in impulse conduction with wandering and altered P waves etc. In the records of the present work almost all phenomena already reported by BÜRGER (1929) have been obtained. It is intended to treat certain electrocardiographic features, especially the fairly regular shift in the axis of the P wave, in a separate paper.

### Summary.

1. Valsalva's experiment was carried out on 25 healthy subjects of whom 13 were smokers and 12 non-smokers before and after smoking of 1—3 cigarettes. In twelve of these subjects control experiments without smoking between two successive Valsalva experiments were carried out.

2. Smoking did not as a rule alter the resting heart rate in non-smokers, but increased it on an average for 16 beats/min. in smokers.

3. The total increase or decrease in heart rate during and after the strain was used as a measure of reactivity. It varied in different subjects, some showing no increase in heart rate at all. Occasionally the type of response obtained in a certain subject could be altered. The smoking tends to decrease the reactivity when effective. A significant correlation between heart rate response and alteration in the height of the P and T waves was found. No correlation between P—Q time and reactivity seemed to exist.

4. The mean electrical axis (and changes caused by strain and by smoking) was calculated with the help of the Dieuaide's chart. The strain tends to deflect the axis rightwards. Smoking did not as a rule affect the mean direction of the electrical field. In 5 instances, however, a leftward shift in the axis was noted. There was no correlation between the size of the heart rate reaction and the rightward deflection of the axis during the strain.

5. Certain special electrocardiographic findings will be treated in a separate paper.

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## Selective Adaptation and the Off/on-ratio of the Retinal On/off-elements.

By

BO GERNANDT.

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The on/off-elements, isolated by the micro-electrode technique in the cat's retina, respond to both onset and cessation of illumination. About 80 % of the elements of that eye belong to this exceedingly complex type of structure (GRANIT and TANSLEY, 1948). The on- and the off-components of such an element may be differentially sensitive to wave-length and, in addition, the ratio of their thresholds to light, the so-called off/on-ratio, may vary over a range of more than 100,000, as demonstrated by the same authors. The highly on-sensitive elements thus have very low off/on-ratios, the highly off-sensitive elements high off/on-ratios. Statistical distribution curves for off/on-ratios have been published by GRANIT and TANSLEY (1948), GERNANDT and GRANIT (1947) and GERNANDT (1948 a).

The contrast mechanism must be somehow organized by the elements capable of responding to both onset and cessation of stimulation and for this reason can hardly be independent of a fundamental property such as the variation in the off/on-ratio. In the present work observations have been made which throw light on this very problem. The thresholds of the on- and off-components have been measured for three wave-lengths, 0.460  $\mu$  (blue), 0.500  $\mu$  (green) and 0.650  $\mu$  (red) before and after selective adaptation with the blue, or green or red Ilford spectral filters.

## Technique and Procedure.

The highly standardized technique of this laboratory was used. This has often been described (*e. g.* GRANIT, 1947). A micro-electrode is inserted into the opened eye of the decerebrate cat, large single spikes isolated, and listened to in the loudspeaker for threshold measurements. The animal is fully dark adapted. Our large Wright colorimeter (see *e. g.* WRIGHT, 1946) has been employed for stimulation.

*Course of the experiment:* The thresholds were determined for three test lights of wave-length 0.460, 0.500 and 0.650  $\mu$ , each value requiring some 8—10 observations, an on/off-element thus around 50 observations. The eye was then illuminated by a tungsten lamp (800 m. c.), run at 2,800° K, in the beam of which was placed one of the three Ilford spectral filters red, green and blue (for their average Transmission curves, see WRIGHT, 1946). After 5 min. continuous illumination the adapting light was interrupted and 5 secs. later the threshold to one of the three test lights was determined. The adapting light was then re-introduced for half a minute in order to maintain selective adaptation, and with such interruptions, the thresholds of the three test lights were determined for a given adapting light.

The eye was dark adapted after the coloured adaptation, and the thresholds followed by repeated measurements until they became normal. Then the same experiment was repeated with another test colour, using the same spike if it could be maintained, otherwise a new spike was located. It is extremely important to make certain that the thresholds return to normal because occasionally the electrode may damage the spot touched. If the thresholds had changed, the experiment was rejected.

Earlier experiments in which several adapting colours were used have not been included in the evaluation of the results.

## Results.

1. *Off/on-ratio and colour.* In an earlier work (GERNANDT, 1948) I had noted that the probability for the off/on-ratio to possess its maximum in the green (0.500  $\mu$ ) was only 0.18 as against 0.47 in the red (0.660  $\mu$ ) and 0.39 in the blue (0.460  $\mu$ ). This suggested that in a certain number of elements (provided that one has a sufficiently large experimental material), the off/on-ratio might be a function of wave-length. In a large number of elements it seems to be approximately constant throughout the spectrum. Fig. 1 A shows the averages for 8 elements in which it varied and it is significant that the variations consisted in an increase of off-sensitivity towards the two ends of the spectrum. This led me to look up earlier measurements, carried out in this laboratory

from other points of view, and in this material too 7 elements with variable off/on-ratio were found. These, when averaged, gave the curve of fig. 1 B, all the more interesting because the experiments from which these elements were taken had been conducted with different purposes in mind. Greater accuracy can, of course, always be expected when the attention is being directed

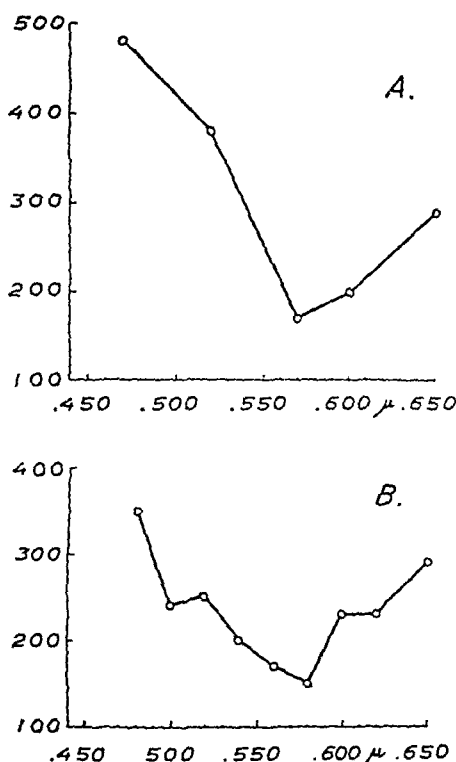


Fig. 1. For full explanation, see text. Ordinates, average off/on-ratios. For the individual curves averaged in A and B the minimum off/on-ratio was given the value of 100 and for the other wave-lengths the values were given as multiples of 100 so as to make it possible to average off/on-ratios from different experiments.

towards one function at a time, but, on the other hand, the agreement between the independent measurements is too good to be fortuitous.

2. *Statistical correlations between on- and off-thresholds.* On a total of 72 elements complete measurements were made of the on- and off-sensitivities at the threshold for the three standard stimuli, 0.650, 0.500 and 0.460  $\mu$ . Regression equations, regression coefficients and correlation coefficients were calculated for this material in terms of log. thresholds.

It appeared that there was no correlation between Blue on and

Red off (correlation coefficient only  $-0.07$ ), Blue off and Red on ( $-0.16$ ), Red on and Green on ( $-0.11$ ) showing that these thresholds had an independent distribution over the elements concerned. GRANIT and TANSLEY (1948) noted that high off-sensitivity to Red ran parallel with high off-sensitivity to Blue. Now it was found that there was a positive correlation of  $0.42 \pm 0.10$  between Green off and Red off, both results thus indicating that highly off-sensitive elements tend to be off-sensitive to all colours so far investigated. There is apparently some fundamental common factor that determines the off-sensitivity of an element.

The most important new finding consisted in the observation that there were definite negative correlations between the on- and off-sensitivities of the two contrasting colours, red and green. Between Green on and Red off the regression coefficient was  $-0.46 \pm 0.10$  and the correlation coefficient  $-0.47 \pm 0.09$ . For Green off and Red on a similar calculation gave a regression coefficient of  $-0.30 \pm 0.08$ , and a correlation coefficient of  $-0.41 \pm 0.10$ . Since the equations were based on the logarithms of the thresholds the negative regression coefficients signify a hyperbolic relation between the two quantities. There is thus in this retina in some elements, at least, a contrast mechanism somewhat obscured by other factors since the negative correlation is of a modest degree.

The negative correlation shows that there must be a certain number of elements in which high sensitivity to red at onset of illumination excludes high sensitivity to green at cessation of illumination and *vice versa*. This is all the more obvious since no similar correlation was noted for respectively green and blue and red and blue. The results presented in fig. 1, suggested something along these lines but, in addition to adding statistical weight to the result, the new observations indicate an explanation in terms of contrast. This may be simply formulated in the following manner: the elements supposed to respond to red at onset of illumination will not in general be identical with those supposed to respond to green at cessation of illumination. If they were identical successive contrast would be impossible since the same element would then have to signal both red and green. The negative correlations thus show that a certain amount of successive contrast is made possible by the organisation of the retinal on/off-elements in the cat's eye.

*Off/on-ratio and selective adaptation.* In all, 40 elements were



Table 1.

Off/on- ratio	Maximal adaptation factor		Off/on- ratio	Maximal adaptation factor	
	on	off		on	off
0.009 .....	23.8		0.31 .....	322	
0.04 .....	18.5		0.35 .....	243	
0.06 .....	63.6		0.50 .....	243	
0.08 .....	142		0.50 .....	550	
0.12 .....	389		0.56 .....		24.1
0.13 .....	25.1		0.69 .....	5.9	
0.15 .....	24.8		0.76 .....		114
0.19 .....	157		2.76 .....		15.1
0.20 .....	244		3.83 .....		244
0.20 .....	114		8.27 .....		233
0.29 .....		248	10.4 .....		218
0.30 .....	34.4				

subjected to selective adaptation with the three Ilford filters. A first glance at this material showed the extreme variability in the adaptive effect. This is best given as an 'adaptation factor' showing how many times the threshold had risen as a consequence of coloured adaptation. These factors varied between one and several hundreds and, in addition, varied with wave-length of test light and adapting light from element to element. Thus there were highly adaptive receptors and receptors which hardly 'felt' the adaptation light and, when several adapting lights were tested with the same element, it adapted strongly when tested with one colour, less so when tested with some other. In this section I propose to neglect all such specific effects. I shall merely discuss the relation between the *maximum* effect of adaptation (independently of colour) and the off/on-ratio. In this respect the results followed a fairly simple and consistent rule.

Table 1 correlates the off/on-ratio with the maximal adaptation factors independently of test colour and adapting colour. It can be seen that, as the off/on-ratio increases, the maximal effect of the adapting light tends to shift from the on-component to the off-component. There are occasional exceptions from this rule but, considering the variability in the properties of the on/off-elements, it is surprisingly well obeyed. It has since been confirmed for 'white' adapting light (GERNANDT, 1948 b).

It is therefore clear that, in general, the adapting stimulus directs its main attack onto the component that is relatively more sensitive to light, so that highly on-sensitive elements become relatively more depressed at onset of stimulation and highly off-sensitive elements at cessation of illumination.

This result can also be formulated in a different way: high sensitivity means low threshold. Thus it seems reasonable to expect the adapting stimuli to cause greater effects on the relatively more sensitive component.

If the adaptive effects were non-selective this would not be much of an advantage for colour discrimination. In the next section it will be shown that, in agreement with earlier observations by GRANIT (1945), the effects in some elements actually are highly selective. The existence of such a state of affairs can already be deduced from the results of the previous section. In those elements for which green on-sensitivity and red off-sensitivity exclude each other, adaptation to green will excite the most sensitive green on-components. These accordingly will become adapted to the stimulus. Similarly the most sensitive green off-components will lose sensitivity on account of their high adaptation factors. But at cessation of illumination the red on- and off-components, shown to belong to other elements, are now ready for full or but slightly depressed performance. The retina will accordingly become red-sensitive at cessation of a period of illumination with green. In this particular preparation, the dark adapted cat, this mechanism is probably carried by a limited number of elements.

*Selective adaptation.* In order to illustrate the variability of the elements with respect to coloured adaptation 4 elements have been selected for each adapting light and presented in Table 2. Three test lights, red, green and blue, are used for each adaptation with one of the Ilford red, green or blue filters. Inasmuch as stimulation is initiated by visual purple decomposition, the red stimulus would be a few per cent only of the other two and the green stimulus would always give the maximal effect. The transmission of the red filter is 0.5 % only at  $0.600 \mu$ , and rises steeply from 4.8 % at  $0.630 \mu$  to 31.3 % at  $0.650 \mu$  (our own measurements).

A glance at the figures shows that the situation is a very complex one.

*Blue adaptation:* the adaptation factors vary for the green test light from 323 to 2.7, for the red test light from 24 to 1.5, for the blue test light from 389 to 4.4. For the first element (uppermost row) the maximum effect is on the green on-component, for no. 2 on the blue on-component, for no. 4 on the green off-component etc. For element no 3, and the green test light, adaptation is minute and the off/on-ratio unaffected as if the ele-

ment had been lacking visual purple. In all other cases there is a change in the off/on-ratio due to adaptation and, on an average, this change is in the direction of unity because adaptation tends to attack the more sensitive component of an on/off-element and thus to diminish the asymmetry of the on/off-element that is expressed by its deviation from unity. Specific colour sensitivity may cause exceptions from this rule. If the characteristic curve of visual purple photosensitivity had been the sole determinant of the properties of the elements the results tabulated above could not have been obtained, unless one assumes that in some elements the state of visual purple in the retina may differ a great deal from its state in solution. This, of course, seems quite probable on recent evidence by DONNER and GRANIT (1948) but need not concern us here.

*Green adaptation:* this filter should have the greatest effect on visual purple photosensitivity and hence give maximal adaptation factors with the green test light which tests the region of maximum visual purple sensitivity. But in element no. 1 the effect is small and maximal for the red test light (on-component) which is tested at  $0.650 \mu$  where visual purple photosensitivity is only about 0.3 %. The green off-component is uninfluenced by adaptation. The use of three test lights makes it possible to obtain some idea about the spectral shift of the sensitivity curve by adaptation. Thus, to use element no. 3 as an example, it is seen that both in the on- and the off-components the adaptation factors in the red are negligibly small compared with those in the green and in the blue. Green adaptation has therefore left the element a great deal more red-sensitive than it was. Similarly, in all cases and for all three adapting colours, a glance at the adaptation factors of the three test lights suffices to give a rough indication of the shift in sensitivity. Very common is a shift towards the red side but there are also elements that have become relatively more sensitive to short wave-lengths as, for instance, in green adaptation, the off-component of no. 1 and in red adaptation element no. 4 (off-component) and no. 1 (on-component).

*Red adaptation:* there is little to add to what has been noted already with the other adapting lights, except that relatively larger effects on the red test light became more common (see elements nos. 1 and 3) and that really high adaptation factors, as in no. 2, were less common. No. 2 was chosen to illustrate that high adaptation factors also could be obtained with the red light

despite its relatively small effect on visual purple photosensitivity. On an average, however, red light caused much less adaptation than green and blue which were about equally effective. With red adaptation an increase of sensitivity was once noted instead of the typical decrease. This may seem improbable but later work with "white" adaptation (GERNANDT, 1948) demonstrated that an increase actually can be obtained and that it probably is due to a shift in the balance between excitation and inhibition maintaining the off/on-ratio. Sometimes this shift during adaptation may overcome adaptive effects.

### Discussion.

The experiments have revealed the existence of an organization of the on- and off-components of certain on/off-elements which follows the pattern prescribed by the rules of contrast between red and green. Other contrast colours have not been tested. Such elements are numerous enough to give a negative correlation of the order of  $-0.46$  between green-sensitivity in the on-component and red-sensitivity in the off-component. This mechanism would be of little avail for contrast if it were not supported by selective effects of different wave-lengths. The experiments have shown that there is, indeed, a very high degree of selectivity in the effects of differently coloured lights on different elements. The variations of the off/on-ratio enter into colour discrimination and contrast by determining the 'point of attack' of the stimulus. If there is high on-sensitivity, the on-component is the one primarily adapting to the light and *vice versa*, if there is high off-sensitivity, the adapting light attacks at 'off'. This means, *e. g.* in the example of red-green contrast, that the most green-sensitive on- and off-components are simultaneously depressed by green adaptation, whilst the red-sensitive on- and off-components (proved to belong to other elements) are left active to take care of contrast.

Let us further consider the nature and significance of the variation in the off/on-ratio. Since it is hardly conceivable that the enormous range of variation of the off/on-ratio represents photochemical differences (GRANIT and TANSLEY, 1948) and since even high degrees of asymmetry of the two components can be compensated by polarizing the retina in an appropriate manner (GRANIT, 1948 a), the off/on-ratio must be largely determined by

Table 2.

No	Test colour	Blue adaptation				Green adaptation				Red adaptation			
		Adaptation factors		off/on-ratio		Adaptation factors		off/on-ratio		Adaptation factors		off/on-ratio	
		on	off	Normal	Adapt.	on	off	Normal	Adapt.	on	off	Normal	Adapt.
1.....	Green	323	15.5	0.12	2.48	17.3	1.0	0.01	0.10	10.0	4.0	1.5	0.38
	Red	23.8	15.2	0.42	0.66	23.8	2.1	0.01	0.15	6.7	15.1	2.19	1.24
	Blue	96.6	39.2	0.41	1.0	13.1	1.3	0.01	0.07	4.0	9.9	3.90	1.56
2.....	Green	23.6	10.0	0.04	0.10	55.0	91.1	0.44	2.68	142	26.6	0.07	0.38
	Red	6.6	1.5	0.23	1.0	23.8	23.3	0.64	0.66	23.8	6.6	0.08	0.28
	Blue	389	5.0	0.10	0.73	308	100	0.41	1.28	24.7	15.6	0.10	0.16
3.....	Green	2.7	2.7	0.05	0.05	100	241	2.44	1.0	6.4	273	4.67	0.11
	Red	4.3	3.6	0.07	0.08	1.5	2.8	6.57	1.55	36.2	159	1.0	0.23
	Blue	4.4	10.3	0.13	0.05	50.0	123	2.17	1.0	4.0	15.5	2.50	0.07
4.....	Green	9.7	170	3.0	0.17	9.0	1.5	0.03	0.17	3.8	2.4	0.27	0.41
	Red	3.6	12.8	2.38	0.66	1.5	1.2	0.17	0.23	2.9	4.5	0.13	0.28
	Blue	9.8	8.2	1.33	1.60	3.9	1.0	0.05	0.20	7.9	2.0	0.03	0.13

the distribution of inhibition and excitation in the synapses. The details of this interesting mechanism of differentiation are not yet fully understood. The present work suggests that this internal re-balancing of the sensitivities to onset and cessation of light is adding emphasis to primary photochemical differences between the receptors according to whether the ganglion cell for a particular colour is meant to serve at 'on' or at 'off'.

Table 2 showed large differences in the adaptation factors for the on- and off-components of many on/off-element. These differences are, of course, immediately reflected in the off/on-ratios, found to the left for each adapting light in the table. Evaluation of the off/on ratio by the higher centres would greatly add to the capacity of colour discrimination in natural vision where an eye hardly ever is quiet. In laboratory experiments, designed to study fixation, LORD and WRIGHT (1948) have detected rapid flicks of 3 to 14 minutes of arc recurring at a frequency of about 2 per sec. for the one observer and 3 every sec. for the other, each flick lasting 0.02—0.03 sec. Such eye movements would tend to induce rapid on/off-fluctuations capable of furthering discrimination.

### Summary.

The threshold of retinal on/off-elements, isolated by the micro-electrode technique in the cat retina, have been determined for three wave-lengths as test light, a red, a green and a blue, before and after adaptation to one of three adapting lights, Ilford spectral green, blue or red.

Statistical threshold correlations were calculated. Blue and red as well as blue and green proved to be independent for on- and off-component, but there was a negative correlation of  $-0.46$  between Green on-components and Red off-components, signifying that a certain number of the elements, responding best to green at onset could not respond to red at cessation of illumination, as would be required by a mechanism of contrast.

Adaptation with the three adapting colours was found to give a number of highly selective and extremely variable effects in terms of the three test lights.

The amount of depression, caused by coloured adaptation, was found to depend on the off/on-ratio in such a fashion that the on-sensitive elements tended to loose sensitivity at 'on', the off-sensitive at 'off'.

For some elements the off/on-ratio was found to increase towards the two ends of the spectrum.

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## Scotopic Dominator and State of Visual Purple in the Retina.

By

K. O. DONNER and RAGNAR GRANIT.

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Most elements, isolated by the micro-electrode technique in the fully dark adapted cat's retina, tend to give a distribution of spectral sensitivity that reasonably well corresponds to the visual purple (V. P.) absorption curve (see *e. g.* summary by GRANIT, 1947) but it has never been examined in detail how closely individual elements of different type, pure on-elements, on/off-elements and pure off-elements fit the V. P. curve. On the theoretical view presented in the above mentioned summary by one of us all variations in photosensitivity of retinal elements, whether due to rods or cones, should ultimately be referable to variations in the chemical linkage between the V. P. chromophore and whatever proteins make up the receptor structure. The different colour curves were all held to be, as it were, "variations on the same structural theme" (GRANIT 1947). Later it was found that it is also necessary to take into account changes by synaptic interaction (GRANIT, 1948 a, b). But such interaction would be of no avail if considerable original differences between the receptors did not exist.

Most interesting light has been thrown upon the photochemical aspect of the question by MORTON and his collaborators. MORTON and GOODWIN (1944) first showed Wald's retinene (see *e. g.* WALD, 1935) to be vitamin A aldehyde. While our experiments were being conducted new evidence appeared. By treating vitamin A aldehyde as well as vitamin A itself with



strong acids MORTON and his collaborators (BALL, COLLINS, MORTON and STUBBS, 1948) produced narrow photosensitive bands corresponding in spectral location with the colour modulators (see *e. g.* GRANIT, 1947). Vitamin A is known to be a complex structure with several conjugated double bonds liable to give coloured photo products. The assumption behind the work of MORTON and his collaborators was that the electrophysiological analysis of colour reception had been "largely concerned with the detection of free radicals derived from the retinenes" (MORTON, 1947). A chromophore of this type, *e. g.* transient orange or retinene, might be expected to exist in states of different probability in the retina, where, in addition, the different receptor proteins themselves provide further variations. For this reason it also seemed to us of great interest to make a detailed quantitative examination of the scotopic spectral properties of a number of single active elements.

Having now for several years applied the micro-electrode technique to the analysis of photoreception in the retina we have ultimately arrived at the stage when it has proved possible, in the most successful cases, to keep one element for several hours under the electrode, in fact, occasionally up to 4–5 hours. A large number of readings can thus be assembled and averaged to give a curve for which one can be certain of minor deviations. The decerebrate cat is the most perfect preparation, so far encountered, and all the work to be presented below has been carried out on that animal. The technique has been repeatedly described (*e. g.* GRANIT, 1947) and we shall therefore only draw attention to some points of procedure adopted to ensure maximum accuracy.

The micro-electrode has been in the nasal corner of the eye where there is no tapetum and the pigment is uniformly black. We have never used less than 2 hours dark adaptation so that in the best animals it has sometimes been possible to reduce the level of intensity of our Wright colorimeter (WRIGHT, 1946) by neutral filters of density 2–3.5 in addition to the wedges regulating the intensity of the spectral stimuli and covering 1–2 logarithmic units. As always in experiments of this type, we have used wave-length  $0.500\ \mu$  as a calibration stimulus and never more than two, sometimes only one other wave-length between each calibration. Changes in the general level of sensitivity of the preparation have thus been corrected for, by always referring them to the corresponding calibration stimuli or distributing a gradual change evenly between two successive calibrations.

In this way from 200–600 individual observations have been made for each spike to establish the correct value of 12–14 points in the spectrum. It might be added that both of us have together carried out

most measurements and, if in disagreement, have repeated the observations until both of us have been satisfied.

## Results.

*Pure on-elements.* These are of particular interest because all the evidence hitherto accumulated suggests that they are the simplest structures in the retina from the point of view of synap-

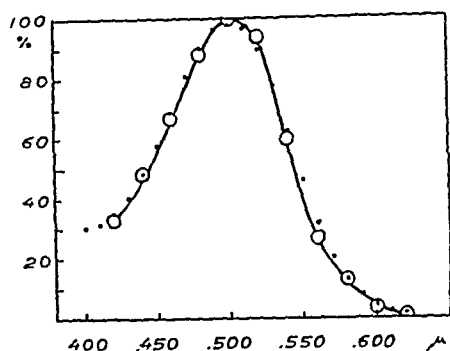


Fig. 1. Dots: in this and figures to follow, standard visual purple curve. Curve drawn through the readings: spectral distribution of scotopic sensitivity of pure on-element. In this and all other figures the spectrum is corrected for equal quantum intensity.

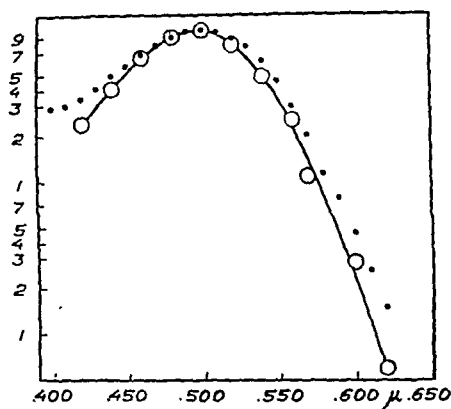


Fig. 2. Average distribution of scotopic sensitivity of four pure on-elements compared with the V. P. curve. Logarithmic ordinates.

tic interaction and also that the receptors combining to give this kind of response are pure rods (GRANIT, 1937, GRANIT and TANSLEY, 1948, GRANIT, 1948, a, b). These elements may therefore be relatively homogeneous, perhaps also with regard to the receptor proteins. For three such elements complete curves were obtained, for a fourth only the top part. In fig. 1 the curve from a very perfect single element is compared with the combined V. P. curve (dots) of LYTHGOE, CHASE and WALD, as averaged by HECHT, SHLAER and PIRENNE (1942). It is seen to be slightly narrower than the V. P. curve showing that the concentration of visual purple of the solutions is slightly different from that of the retina. That such differences must occur was pointed out by one of us (GRANIT, 1941) as well as by HECHT, SHLAER and PIRENNE (1942) and by BALL, COLLINS, MORTON and STUBBS (1948). The two other curves were still narrower than the V. P. curve and the average of all of them is given in fig. 2, this time plotted on loga-

rithmic ordinates to show the deviations at the base of the curve more clearly.

*The on/off-elements.* A number of on/off-elements behaved like the pure on-elements. Thus there can be no doubt but that this particular distribution of sensitivity represents a highly probable state and concentration of visual purple in the retina.

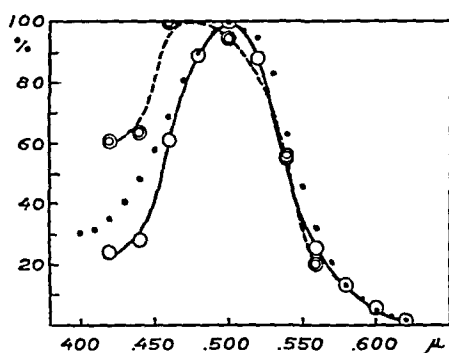


Fig. 3. The dotted curve drawn through readings referring to the on-component of an on/off-element, the one in full to the off-component of another on/off-element.

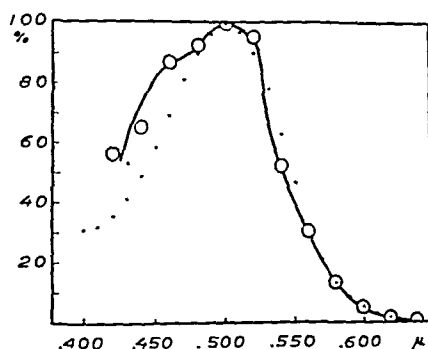


Fig. 4. Averaged scotopic values of on/off-components of a relatively off-sensitive element observed during  $4\frac{1}{2}$  hours.

Let us now inspect some other types of curve. Fig. 3 shows two elements from the same retina, both of the on/off-type. One of them (drawn in full) seems to be the characteristic slightly narrower V. P. curve already described but the other one very definitely expands to the left to give a very much higher sensitivity to blue light than seen hitherto. One might, of course, say that this deviation is due to 'blue cones' but this, after all, is merely a way of expressing that one has found it necessary to assume something else than the standard curve for visual purple. The extremely high sensitivity of the 'blue expansion' does not agree very well with our conventional views on the properties distinguishing rods from cones. For this and several other reasons, given above, we prefer to speak of another probable state for visual purple in the retina signifying that the combination of the free radicals within the chromophore + receptor protein has turned out slightly different from the standard type of fig. 2.

Internal filters might be mentioned as a possible explanation but there is no evidence for such filters in the cat's eye and haemoglobin would rather tend to suppress humps in the short wavelengths.

Fig. 4 is another specimen of this 'blue-sensitive' form of visual

purple. This element was kept  $4\frac{1}{2}$  hrs. under the electrode and 650 observations were made on both 'on' and 'off' for which the values have been averaged. The high value for  $0.420 \mu$  should be noted. This point was high also in fig. 3. We thought these values due to stray light from which the Wright colorimeter is not quite free at maximum intensity but then several of our highest values

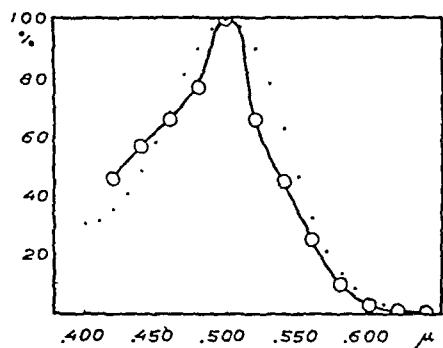


Fig. 5. An extremely high-sensitive pure off-element. According to the protocols the spike was particularly easy to measure.

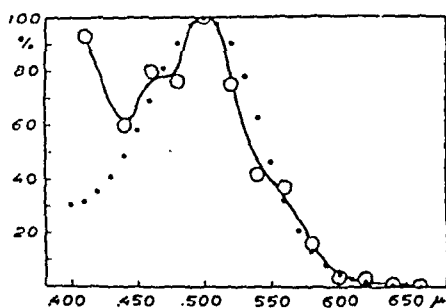


Fig. 6. Averaged scotopic values for on/off-components of an on/off-element.

for  $0.420 \mu$  were obtained with so many filters in the light beam that the proportion of stray light necessarily became negligibly low, in our opinion subthreshold. This view was confirmed when (as in fig. 3) low and high values at  $0.420 \mu$  were obtained on the same animal but differed systematically for two different elements.

A very interesting curve is the one of fig. 5, narrow at the top and at the red end, expanding towards the blue and violet. The element of fig. 6 has the same narrow top and is similarly 'excavated' to the right but again expands towards the blue end. In the blue and violet it shows two humps. The hump at  $0.420 \mu$  is the highest ever seen in these experiments. It reappeared in four measurements at both 'on' and 'off'. In all, there were 415 observations and the values for the on- and off-components were averaged, as it is rather difficult to obtain the high degree of certainty, aimed at in this work, for *both* components separately.

Fig. 7 shows the averages of all the scotopic curves measured in the work of GRANIT (1945), plotted on logarithmic ordinates to illustrate that in this lot there happened to be a large number of elements which were relatively red-sensitive. The thin curve is the visual purple curve. A number of such elements were also encountered here and one is given in fig. 8 as the curve drawn in full. The plot is non-logarithmic but nevertheless shows very

clearly that the experimental curve lies above the V. P. curve (dots) in the red. With the rat similar rises in the red were shown to be due to some less adaptable substance than the rest of the curve (GRANIT, 1941, 1947) and for this reason it was decided to remove part of the visual purple by slight light adaptation. A white light of 800 m. c. was used together with a neutral filter of

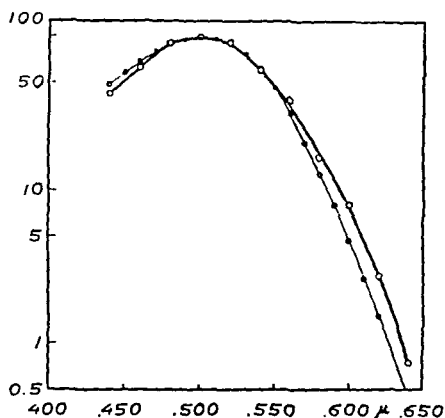


Fig. 7. Logarithmic plot of average scotopic curve from the work of GRANIT (1945). The thin line joins the dots representing the V. P. standard.

density 2.4, thus a modest light adaptation. The result is shown in the dotted curve. There is a very large rise in the red suggesting that the particular free radicals or protein linkages responsible for high red-sensitivity represented some different less adaptable state of visual purple or, again, what in conventional terms would be called cones. There is other evidence of inhomogeneity. The blue region also shows relatively too high sensitivity. It

should be noted that the light adapted curve is plotted on a percentage basis and that the top at  $0.500 \mu$  maintained itself as the maximum though in this region the curve became a great deal narrower than before and, on an average, 300 times less sensitive than in the dark. The modest adapting light was kept on all the time and merely interrupted for a few seconds for each observation. An initial period of 10 min. uninterrupted light adaptation preceded the beginning of the work during which a gradual fall took place that was followed by taking alternately the calibration wavelength and the one to be determined.

An exactly similar experiment was performed with the element presented in fig. 9 in which again the curve drawn in full represents full dark adaptation and the dotted curve the result after modest light adaptation. This time the inhomogeneity of the curve merely expressed itself in the blue and violet region where there is a hump with a maximum at  $0.460 \mu$ . It is not our intention now to explore the possibilities of this method further. The curves shown are merely specimens chosen to illustrate salient points.

Both white and coloured adapting lights have recently been used by GERNANDT (1948) in somewhat differently designed ex-

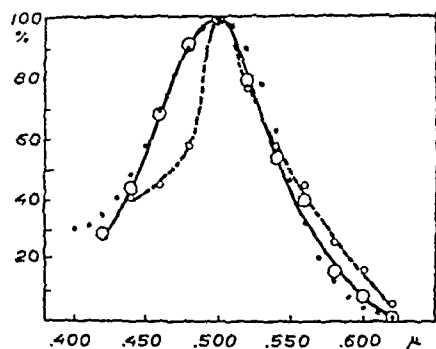


Fig. 8. The scotopic curve, drawn in full, refers to the off-component of a slightly more off-sensitive on/off-element. The dotted curve represents the properties of the same element after modest light adaptation, as explained in the text.

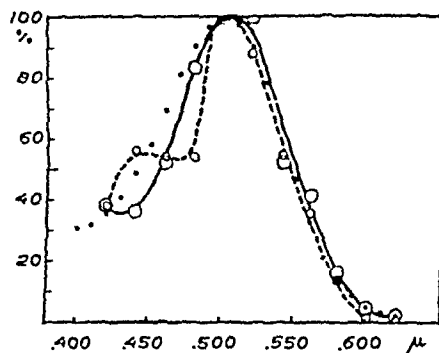


Fig. 9. Same experiment as in fig. 8 with another element.

periments. He did not explore more than three points, one in the red, one in the green and one in the blue but, instead, studied a very large number (40) of well isolated spikes. One of the most striking features of his experimental material was the incredible amount of variation in the adaptability, both general and specific (or selective with respect to colour).

### Conclusions.

As pointed out above, we are not fully convinced that it serves any useful purpose to lay the main emphasis of thinking and further research on concepts such as 'rods' and 'cones'. The important work of MORTON and his colleagues has clearly shown that other modes of theoretical thinking at the moment are more profitable and lead to concepts better capable of furthering the analysis of colour reception.

Our results as well as those, mentioned above, by GERNANDT (1948), interpreted along such lines, suggest that some highly asymmetrical chromophore of visual purple (vitamin A aldehyde?) exists in the retina in several possible states of different probability, partly, at least, dependent also upon the receptor proteins which from extreme 'rods' to extreme 'cones' at the other end of the scale well may differ considerably from one another. When visual purple is extracted by the various methods of extraction the most probable form is obtained whilst all the del-

icate linkages, formed by the free radicals of the chromophore, which cause the resonance responsible for colour variations, are destroyed. It is, at this stage of our knowledge, an entirely secondary matter whether we call the substances rod-substances or cone-substances. We may just as well speak of attempts by nature to elaborate a mechanism of colour vision by trying to fit the chromophore to various proteins. Under certain circumstances (in cone-eyes, such as the snake, always; in mixed eyes, after strong light adaptation) modulators are turned out, in other cases there is the modulation of the dominators, previously demonstrated for the photopic dominator (see *e. g.* GRANIT, 1947) and now for the scotopic one which, on the strict rod-cone concepts, never could have had the properties described above.

This work also demonstrates a new region of modulation at  $0.420 \mu$ . Unfortunately our spectrum does not give enough light further out in the short wave-lengths. The other regions in which humps have been seen are known from previous work (see *e. g.* GRANIT, 1947) on the modulators. 'White' or coloured adapting lights may apparently be used to analyze the variability of chromophore-protein linkages because under the influence of light adaptation the chemical bonds concerned seem to show up their statistical preferences in the spectrum.

The curves of the substances produced in the receptors are subjected to further transformation by excitation and inhibition in the synapses, as shown by experiments of another type (GRANIT 1948, a, b). The latter processes do not create anything new, to judge by the fact that the modulators obtained by such secondary methods agreed with those obtained previously by direct measurements. But they must add to the discrimination, particularly by supporting contrast.

The standard scotopic luminosity curve of the human eye agrees fairly well with the V. P. curve of fig. 2 for the pure elements. This may signify that such elements have been responsible for it, say, by being in the majority at the absolute threshold, but it can also mean that differences simply have been smoothed out by averaging the variations observed instead of analyzing them.

### Summary.

Single spikes have been recorded by the micro-electrode technique from the retina of the fully dark adapted decerebrate cat, the aim being to determine the curve of the scotopic dominator with a maximum degree of accuracy in order to find out whether it corresponds with the visual purple (V. P.) distribution of sensitivity as known from work on retinal extracts.

The pure on-elements and some of the on/off-elements gave a curve slightly narrower than the V. P. curve (*e. g.* figs. 1 and 2).

Some on/off-elements behaved differently. There were humps in the long and short wave-lengths in the regions where previously modulators have been noted. In some elements unexpectedly large values were obtained at  $0.420 \mu$  (*e. g.* figs. 3—6).

It is concluded that, since the V. P. chromophore would be a complex structure with several conjugated double bonds, it may exist in the retina in states of different probability depending upon the free radicals and the receptor proteins.

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## On the Correlation between Hyaluronidase Content and Spermiogenesis in the Testes.

By

EINO KULONEN.

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It was observed in 1928 that testis extract has a marked effect upon the permeability of tissues in the capacity of a "spreading factor" (DURAN-REYNALS, 1928; McCLEAN, 1930, 1931). Injections of pathogens or staining media become diffused much more rapidly if mixed with testis extract. A similar property has been found in extracts prepared from a number of pathogenic bacteria (DURAN-REYNALS, 1933), for instance from species of the *Clostridium* which cause *e. g.* gaseous gangrene (McCLEAN, 1936), in snake and bee venom (DURAN-REYNALS, 1939) and in leech extract (CLAUDE, 1937). Certain tumors have also been reported to possess a spreading factor (BOYLAND and McCLEAN, 1935). It has been encountered in general under circumstances which require rapid movement of fluids or cells within the tissues. Unsuccessful attempts have been made to chemically differentiate the spreading factor from hyaluronidase, *i. e.* from the enzyme which decomposes the mucous hyaluronic acid polymerized from glucosamine and glucuronic acid (CHAIN and DUTHIE 1939, 1940; HAHN 1943).

The considerable hyaluronidase content of the testis, particularly as the sperm contains the same enzyme (HOFFMAN and DURAN-REYNALS, 1931), gives direct reason to assume that this substance is connected with the function of propagation. An enzyme acting upon the cervical mucus is also present in the sperm, but it is not hyaluronidase (CHAIN and DUTHIE, 1940).

Already in 1912 LONG observed that the cells of the corona radiata surrounding the ovum are bound together by means of a gelatinous, transparent substance. If a rabbit ovum is permitted to mature undisturbed, from 6 to 8 hours will pass before the cumulus cells become detached, which is conditional for the final maturation of the ovum. During this period a protein-containing membrane is formed on the surface of the ovum; this membrane impedes the entry of the spermatozoon (cf. SWYER, 1947). YAMANE (1930, 1935) studied the action of the sperm upon the corona radiata and found it to possess a thermolabile factor which effects the rapid decomposition of the corona radiata and the maturation of the ovum. This factor was not species specific. As similar results were obtained by YAMANE with pancreatic trypsin, he regarded the action as being directed against proteins. However, it cannot be a proteolytic enzyme, as in such a case it would exert an injurious action also upon spermatozoa and other cells.

Recent studies have explained this reaction with the help of the hyaluronidase of the sperm. McCLEAN and ROWLANDS (1942) proved in their experiments with rats and FEKETE and DURAN-REYNALS (1943) with mice that hyaluronidase produced by various methods decomposes the corona radiata in about 2 to 3 hours, and that the action is proportional to the enzyme concentration. They presumed that the empiric minimum number of spermatozoa is required for fertilization because only then is the necessary hyaluronidase content attained, even if a single spermatozoon is sufficient to effect the fertilization itself. Thus ROWLANDS (1944) was able to indicate that by addition of hyaluronidase to diluted sperm, fertility was improved to some extent.<sup>1</sup>

There exist so far very few studies on hyaluronidase in the sperm. JOËL and EICHENBERGER (1945) have studied the sperm of 51 persons and report that the hyaluronidase concentration is in some degree proportional to the spermatozoa count. Hyaluronidase was totally absent in cases of aspermia and azoospermia. If the sperm count was very low the hyaluronidase content was relatively higher. These investigators draw the conclusion that hyaluronidase is apparently a product of the activity of the tu-

<sup>1</sup> During printing of the present paper, AUSTIN has published (Nature 1948. 162. 63) observations according to which decomposition of the corona radiata would not be indispensable for fertilization. However, in my opinion local action of hyaluronidase or possibly of some other so far unknown polysaccharase bound with spermatozoa may be required.

bulus epithelium, and add that its presence is independent of whether spermatogenesis is complete or not; however, they do not prove their statement more definitely. They observed that the enzyme is present both in the spermatozoa and the sperm plasma and that the amount of enzyme is dependent solely upon the number of spermatozoa present. WERTHESEN and collaborators carried out analogous experiments and reached similar results (WERTHESEN, BERMAN, GREENBERG and GARGILL, 1945). They suggest that the spermatozoa count should be replaced by a hyaluronidase test, which is easier to perform. LEONARD, PERLMAN and KURZROK (1946) have carried out experiments with bull sperm but have found the correlation mentioned above to hold true only with the same individual. The most extensive studies on the subject have been made by SWYER (1947), who for his part establishes an accurate correlation between the sperm count and the hyaluronidase content with man, rabbit, bull and hog. No definite rule could be observed with the dog. The sperm of the cock contained only a minor amount of hyaluronidase; this find SWYER connects with the fact that birds have no corona radiata. He found that most of the enzyme occurs in the spermatozoa and he therefore assumes on the basis of his experiments that the enzyme is released when the spermatozoa come into an environment which previously contains no hyaluronidase and in which the sperm fluid becomes diluted. The contents of the oviducts and the hyaluronic acid have no effect on the release of hyaluronidase.

Systematic studies have not been carried out on the testes themselves. In 1938 SPRUNT, HOOKER and RAPER indicated that hyaluronidase is not produced in undeveloped testes and that the amount is reduced in both congenital and artificially produced cases of cryptorchism. Histologically established atrophy and degeneration had developed simultaneously. These authors regard spermatogenesis as necessary or even conditional for obtainment of high concentrations of the enzyme. KURZROK and co-workers, who also have studied the testes, have in similarity with SWYER observed that no hyaluronidase is present in the testes of the cock and that with the rat the amount decreases after removal of the hypophysis. In this connection it may be well to point out that the sex hormones in themselves possess no spreading action. The production of hyaluronidase is reduced in the testes of famished animals (TOBIN, BERGENSTAHL and STEFFEE, 1948).

DURAN-REYNALS has carried out experiments with Lindbergh's apparatus to determine whether release of the enzyme can take place without decomposition of the cells, but as he was unable to retain the cells intact he does not give further information. In the light of the studies on sperm an affirmative reply is probably the correct one.

The investigations described below were prompted by the observation, made in connection with the production of hyaluronidase preparations in 1946, that all testes are not of equal value. The purpose of this study has been to find out whether parallelly performed hyaluronidase determinations and histological examinations could shed further light upon the origin of hyaluronidase and upon its release from the testes and from cells in general.

### Method.

The material comprised 22 bovine testes obtained from a slaughterhouse. The specimens were deep-frozen ( $-12^{\circ}\text{C}$ ). Connective tissues with the exception of the tunica albuginea were removed and the testes weighed. A histological specimen was prepared from near the outer surface of each testis by fixation with sublimate-acetic acid according to Lang's method, mounting in paraffin in the usual manner, and staining with Hansen's hematoxylin. Of some of the testes frozen sections were also prepared by the gelatin method and fat-stained with sudan. The dry substance was obtained by storage at  $110^{\circ}\text{C}$  for 48 hours.

For the enzymatic test the tissue was ground in the Latapie mill to make measurement by pipette possible. To 2 ml of specimen the same quantity of  $n/10$  acetic acid and 1 drop of toluol were added, after which the mixture was extracted and centrifuged.

As substrate was employed hyaluronic acid prepared from umbilical cord and buffered with phosphate to pH 6.8; a sufficient amount of NaCl was added to bring the concentration in relation to NaCl to 0.2 mol. and the viscosity in relation to water to 1.75. According to MADINAVEITIA and QUIBELL (1940) the half-value time of viscosimetrically observed depolymerization is directly proportional to the enzyme concentration. This, it is true, is not quite accurate according to SWYER and EMMENS (1947), but the error is not great. The substance to be tested was added to the substrate in the ratio 0.05 : 3.0, but for control purposes tests were carried out also with other amounts of enzyme. The results were found to conform within an error limit of 10 per cent.

To determine the possible effect of autolysis, tests were made by dividing the specimens into three portions, to all of which  $n/10$  acetic acid, pH 6 phosphate buffer, and toluol were added. One of the portions was placed in the thermostat at  $+37^{\circ}\text{C}$  and the second in the refrigerator

Tab. I.

T sec.	1 000 T	Dry subst.	Weight g	Histological findings				R
				sp.	spg.	pl.	Remarks	
∞	0	14.5	35	—	—	—	Tubuli contain simple epithelium	
790	1.25	14.0	30	—	(+)	—	Epithelium partly simple	
580	1.75	9.8	70	(+)	(+)	—	Tubuli small but cells abundant	++
475	2.10	11.6	40	—	+	—	— » —	+++
450	2.25	11.8	40	(+)	+	(+)	— » —	++
270	3.70	11.1	315	+	+	+	Spermatoids abundant	+
230	4.35	11.5	45	++	++	++	Tubuli usual, fair amount of cells	
210	4.75	11.8	85	+	+	+	Tubuli large	
180	5.55	9.8	115	+	++	+	Spermatoids abundant	
175	5.70	10.1	75	++	++	++	— » —	
165	6.05	10.9	110	++	++	++	— » —	
160	6.25	10.8	110	++	++	++	Tubuli filled with cells	
155	6.45	10.2	160	++	++	++	— » —	++
155	6.45	—	135	++	++	+	— » —	
150	6.55	11.2	180	+++	++	+++	— » —	
120	8.35	10.5	130	+++	+++	+++	Tubuli large, mature spermatozoa	
115	8.70	10.8	105	+++	+++	+++	— » —	+
115	8.70	9.8	240	++	+++	++	— » —	
105	9.55	11.2	115	++	++	+++	— » —	
60	16.6	11.8	145	+++	+++	+++	— » —	
65	17.0	12.8	195	+++	+++	+++	— » —	
65	17.0	12.5	135	+++	+++	+++	— » —	

T = time for decline of viscosity to half value

sp. = spermatozoa

spg. = proliferations of germinal epithelium

pl. = tubuli contain substance which takes fibrinoid stain

R = tissue takes fat-stain (Leydig's cells)

for 48 hours. From the third portion the solid substances were centrifuged off, after which it was also placed in the thermostat in order to determine possible destruction of the enzymes.

## Results.

The autolysis tests revealed an approximately equal enzymatic intensity in all three portions of the specimens. Grinding is believed to have been so thorough that all cells were broken and therefore when the enzyme went into solution a process of actual decomposition did not take place but rather a mechanical release.

Other test results are presented in the table. The values obtained indicate great variation in the enzyme content. In regard to its relation to the size and the amount of dry substance of the testis, mutual correlation is not unconditional, yet there exists a definite

trend also in this respect. In the maximum values for hyaluronidase the amount of dry substance is slightly higher than usually.

The findings at histological examination will be seen in detail from the table. Special attention was paid to the spermiogenesis and to the spermatozoa count. Were it possible to obtain testes in which spermiogenesis is in the same phase throughout, conclusive observations could be made, whereas all stages of development are simultaneously present in most animals. Correlation was plainly evident between the spermatozoa count and the activity of the spermiogenesis on the one hand and the hyaluronidase content on the other hand. The tubuli of those testes in particular in which the greatest amount of enzyme was present contained an abundance of fluid with mature spermatozoa and spermatoids. "Tassels" of maturing spermatozoa adhering to Sertoli's cells were found mostly in those testes which contained a fair amount of hyaluronidase. It would be of interest to learn whether or not hyaluronidase is present in the spermatozoon already at this stage; however, a reliable answer to this question cannot be given on the basis of tests of the type here described. There were a few testes in this material in which no mature spermatozoa could be established but an abundance of cells was found in the epithelium. These cells contained hyaluronidase, even if in small amounts, but no enzyme was present if the epithelium was simple. The fluid in the tubuli, which forms a part of the sperm "plasma", was very abundant in the testes with hyaluronidase content and it even seemed to penetrate between the cells of the germinal epithelium. With men over 25 years of age, degenerative forms are always found at some point according to STIEVE (1930), and the forms in which differentiation is the farthest advanced suffer first. This may be the reason for the low content of hyaluronidase in some large-sized testes despite an apparent abundance of cells. Of course a large number of cells need not in itself be conducive to the production of spermatozoa; this is apparent already from the fact that before puberty the tubulus epithelium proliferates, whereas spermiogenesis is deficient.

Fat-staining disclosed no special findings. The interstitial layer, it is true, was more abundant at the points where spermiogenesis only was incipient, but this inverse relation may have been due merely to the well known relation of Leydig's cells to the activity of the germinal epithelium, and the interstitial cells probably have no direct relation to the production of hyaluronidase production.

## Discussion.

On the basis of papers on sperm studies of which I learned after the practical work already partly had been carried out, the existence of a correlation between the hyaluronidase content of the testes and the condition of the tubulus epithelium could be expected. The question of the stage at which the maturing sperm cells receive their enzymatic property is of interest also from the general point of cell secretion. The results seem to indicate that already an incipient spermiogenesis is sufficient but that maximum values are attained only if and when mature spermatozoa have been produced. It cannot be regarded *a priori* that sperm cells would differ from other rapidly growing cells in the organism, even if nature in some way has noted their special function, for hyaluronidase does not occur in birds, who have no benefit of it. MEYER (1947) has noted that the presence of hyaluronidase can be autolytically indicated in the skin<sup>1</sup>, which in the adult also contains rapidly growing hair follicle cells. It could be assumed that all rapidly growing cells would contain a "spreading factor" which could be brought out in some manner, whereas from some cells it would be freely released into the environment. The amount of enzyme of course varies greatly. It has been demonstrated that with some bacterial strains enzyme production is greatly stimulated if hyaluronic acid is present in the culture medium. In the sperm cells the hyaluronic acid content of the environment is of no significance for the release of the enzyme, as was already stated. Nevertheless the capacity of the sperm to produce hyaluronidase might be compared to the capacity of the bacteria for enzymatic adaptation. There is every temptation to explain the process of release of hyaluronidase on the basis of the findings made on sperm by SWYER and by JOËL and EICHENBERGER, *i. e.* that the higher the sperm dilution, the more enzyme is released into the environment. My own findings also indicate a marked increase in the amount of fluid in the tubuli and the presence of the greatest amount of fluid in those testes which have a very high hyaluronidase content. However, it probably is nearer the truth that these features are connected with the active metabolism required by spermiogenesis.

<sup>1</sup> GLICK and GRAIS (Arch. Biochem. 1948. 18. 511) have shown this finding to be very doubtful.

LEONARD's observation that with rats from which the hypophysis has been removed the hyaluronidase content of the testes is lower is probably explained by the altered spermiogenesis. The same apparently is true of undernourished conditions.

The chemical structure of the enzyme is not known, nor whether it is present in the plasma or in the nucleus of the cells. It is true there are some older findings according to which given substances resembling protamine are stated to have a spreading effect. The low plasma content of the spermatozoa also seems to support the theory that the enzyme originates in the nuclear components.

The significance of the anatomic structure of the spermatozoa is entirely unknown. During spermiogenesis the protoplasma continuously decreases in amount. There is an abundance of nuclear substance near the outer surface of also other hyaluronidase-containing cells and of bacterial and tumor cells and it therefore can easily be released, contrary to what the case is in the cells of the skin, which must first be autolytically decomposed. The correlation between hyaluronidase and the other enzymé systems of the sperm, primarily the glycolytic and proteolytic systems, has not been discovered.

It is generally known that the sperm requires a given length of time to attain fertility and that the first cells that reach the oviduct are not capable of fertilization. These facts probably could be explained by the theory that release of hyaluronidase is necessary for attainment of fertility. It does not seem likely that free enzyme would ascend as such into the oviducts but rather that the release of the enzyme occurs there (SWYER, 1947).

From the clinical point the use of hyaluronidase in sterility originating in oligospermia, and particularly in astenospermia, in which, however, the cells probably often are defective also otherwise. The artificial impregnation of cattle may derive benefit from the use of hyaluronidase.

### Summary.

Investigations on the relation between morphological factors and the presence of hyaluronidase in bull testes are described. A very close correlation has been found to exist between the activity of the spermiogenesis and the hyaluronidase content. It would seem, however, that complete spermiogenesis is not required for the production of hyaluronidase. Thus the presence



of hyaluronidase probably is a condition for fertility, but its occurrence is not *per se* an indication of the capacity to fertilize.

The size and the dry substance content of the testis possess a certain correlation with the hyaluronidase content.

The formation of hyaluronidase in cells in general is discussed.

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From the Dept. for Experimental Histology of the Karolinska Inst.  
and the Wenner-Gren Institute, Stockholm.

## Studies on the Rôle of the Cerebrospinal Fluid in Brain Metabolism as Measured with Radioactive Phosphate.<sup>1</sup>

By

L. BAKAY and OLOV LINDBERG.

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Since the classical experiments of GOLDMAN (1913), it has been known that the brain deserves, from the standpoint of permeability, a special consideration. There seems to exist a "barrier" system which provides that only certain substances in suitable amounts may pass from the blood to the brain. Thus in experiments with dyestuffs it is known that acid dyes do not enter from the blood into the brain and with studies using vital dyes, the brain parts remain uncoloured, but by injecting the dyes directly into the cerebrospinal fluid, the brain becomes dyed.

SPATZ (1934) pointed out that after parenteral or intravenous injection of trypan blue, for example, the tissues of the organism, including liver, were dyed blue, for the dyestuff was withdrawn from the blood stream to stain the cells. The brain on the other hand remained undyed. This can be explained by assuming that no trypan blue was withdrawn through the brain capillaries.

Investigations have also shown that the blood-brain barrier is not everywhere impermeable to acid substances of large molecular weight. After intravenous injection of trypan blue, the greater portion of the brain remains uncoloured, but shows a distinctly outlined coloration in some places; in these sites the barrier is clearly permeable. These places are the pituitary gland, infundib-

<sup>1</sup> This work was supported by a grant from the National Research Council.

ular region, recessus opticus, paraphysis, pineal body, several small parts of the medulla oblongata and the plexus chorioidei (SPATZ 1934).

The penetration of various ions from the blood into the cerebrospinal fluid has also been studied, with the help of radioactive isotopes. GREENBERG (1943) et al. injected different kinds of isotopes into the blood vessels of dogs and then determined them in the spinal fluid drained from the cisterna. The curves obtained were similar in all cases. The concentrations in the plasma decreased proportionally to increase in the spinal fluid. The penetration, however, proceeded very slowly, much more slowly than can be explained by filtration, which thus strengthens the plexus secretion theory. VISCHER and CARR (1944), as well as WANG (1948), obtained similar results.

Regarding the blood-brain barrier, it is known that intravenously injected  $P^{32}$  appears in the brain in only vanishingly small amounts. This has been supported by our own experiments. HEVESY (1947), BORELL and ÖRSTRÖM (1945) found, after intraperitoneal injection of  $P^{32}$ , the radioactivity in the brain to be very low except for certain portions. These, the pineal body, pituitary gland, plexus chorioidei, and part of the hypothalamus, showed a very high specific activity. These portions, therefore, differ fundamentally from the other parts of the brain.

The relation between the cerebrospinal fluid and the tissue fluid of the brain has been studied for many years but is still largely regarded as an unsolved problem. One point of investigation is to decide whether spinal fluid is identical with the extracellular fluid of the brain or not, and if the latter were the case, what relation exists between the two systems. The problem of brain metabolism is also closely related to this question. The two different extremes regarding the rôle of spinal fluid in the metabolism of the brain are reflected in the concepts of HAUPTMANN (1925) and of LANGE (1935). According to the former, the metabolic turnover of the brain takes place exclusively through the spinal fluid ("Weg über den Liquor"). The latter on the other hand regards the spinal fluid as a protective structure ("totes Füllmaterial") against possible external injury.

From these data, it was thought interesting to study the metabolism of the brain with the help of  $P^{32}$ , an especially suitable isotope for this purpose. In order to by-pass the biological filters (blood-brain and blood-spinal fluid barriers) by which the brain

is isolated to some extent, the isotopes were injected into the cisterna magna, thus coming directly into the cerebrospinal fluid circulation.

It follows from the nature of this study that besides the consideration of brain metabolism, its objective is a greater understanding of the circulation and resorption of the cerebrospinal fluid.

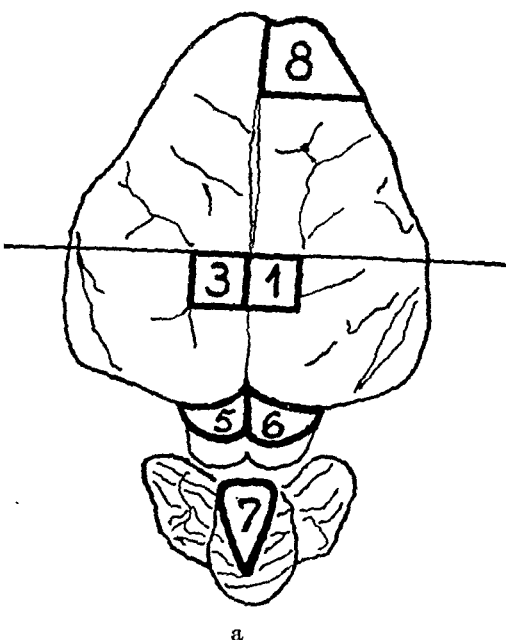
## Methods.

The rabbits used for the study were anaesthetized with intraperitoneally injected narcotal, the action of which was supplimented with ether inhalation. Fourteen experiments were carried out. When the animals were deeply relaxed, the cisterna cerebello-medullaris was punctured. To avoid excess pressure in the subarachnoid space, a volume of the clear cerebrospinal fluid was withdrawn, equal to the volume which was later injected. In case the fluid was contaminated with blood, the experiment was discontinued.

The withdrawn cerebrospinal fluid was replaced with an aqueous solution of radioactive sodium phosphate (usually 0.1 ml. of solution) having a radioactivity of 5 microcuries. The data obtained when the amounts of injected radioactivity differed from this were recalculated to what they would have been at the 5 microcurie level. In our counting chamber, this was equivalent to 750,000 impulses per minute.

Immediately after the injection, blood samples from different blood vessels were taken, each with a different needle to avoid contamination of samples with strong radioactivity. Blood samples were taken at intervals of 2—5 minutes, and with longer experiments, at intervals of 10 minutes.

The animals were killed at 8, 18, 28, 35, 38, 45, and 62 minutes and at 4 and 24 hours after injection of  $P^{32}$  by a probe in the heart or by means of air emboli. The intact brain was removed undamaged and rinsed with isotonic phosphate buffer and thereafter with a strong stream of running water in order to completely remove the highly radioactive cerebrospinal fluid from the brain surface. The cerebrospinal fluid which remained between the lobes was washed out by rinsing the lobe system under a strong stream. The brain was then frozen in carbon dioxide snow. The  $P^{32}$  content of the different parts of the brain was determined. The brain was divided according to a scheme carefully arranged in advance (figs. 1 a and b). These parts, namely the frontal pole, cortex convexitatis, cortex basalis, subcortical zone, corpus striatum and central white substance, the upper colliculi of the corpora quadrigemina, and also a fragment of the vermis of the cerebellum, were cut out of the frozen brain with special knives and chisels to avoid the post mortem movement of the tissue fluids and  $P^{32}$  content. The parts were weighed, and showed very little weight differences in the various experiments. Specimens were taken from both halves of



b

Figs. 1 a and b. Figures showing how the brain is sectioned and how the portions are numbered.

the brain, and were separately analysed in duplicate to thus have a check on the analytical error and biological variation.

The specimens taken for analysis amounted to 8–10 % of the total weight of the brain; the remainder, about 90 %, with the pineal body and pituitary gland removed, was analysed as a separate sample.

Phosphate was determined by the usual method according to Fiske and SUBBAROW. The content of radioactive phosphate in the different phosphate samples was always determined in the form of crystallized  $\text{MgNH}_4\text{PO}_4$  which was filtered by suction into small aluminum dishes. To keep the amount of phosphate constant, a certain amount of inorganic phosphate was added to the sample before precipitation. Cf. LINDBERG (1946).

To determine the radioactive phosphate content, the different brain portions were decomposed in 1 ml. of concentrated sulfuric acid and oxidized with a few drops of concentrated nitric acid. When the oxidation was complete, the sample was diluted to 50 ml. and aliquots were taken for phosphate and radioactivity determinations. In this way, the possible manipulative loss during oxidation was significantly reduced. The number of radioactive impulses were calculated per mass of phosphate.

For the measurement of the exchange between organic and inorganic phosphate, the brain (excluding the epiphysis and the pituitary) was ground with 10 % trichloroacetic acid, and the organic as well as inorganic phosphate was determined in the clear filtrate.

The relation between the number of radioactive impulses per phosphate atom (the specific activity) for the organic-bound phosphate, and the same quotient for the inorganic phosphate becomes a measure of

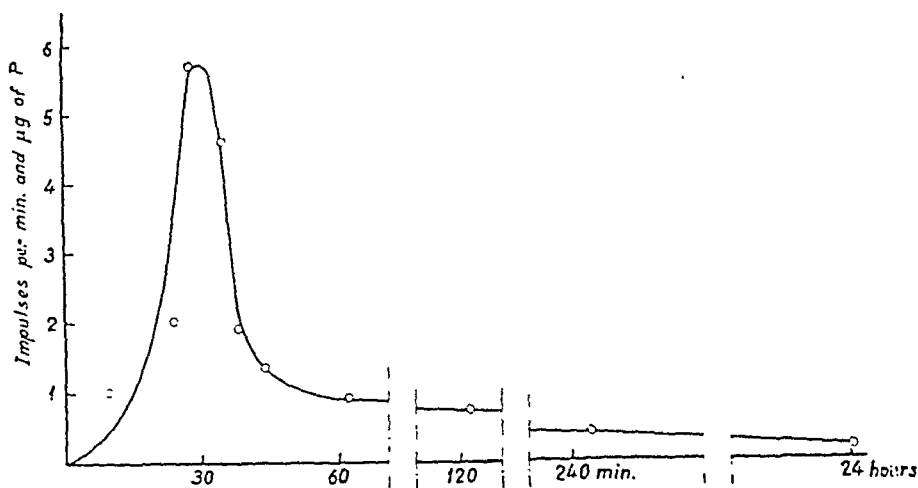


Fig. 2. Figure showing the variations of radioactivity in brain after intracerebrospinal injection of 5 microcurie radioactive sodiumphosphate.

the speed of phosphorylation. In order to get a uniform check in every experiment, these quotients between specific activities were also determined for liver. Cf. HEVESY (1947).

### Experimental.

After injection of  $P^{32}$  into the cerebrospinal fluid in rabbits, the content of the radioactive phosphate isotope increased in the brain (Fig. 2) and its different parts as shown in Fig. 3. The radioactivity values were calculated as the number of impulses per microgram of total phosphorus. The analysis of the total phosphate content of the brain shows that 100 g of substance contains 300 mg of phosphorus of which 69 mg is soluble in trichloroacetic acid. When the curve passed its maximum after 30 minutes, 20 % of the injected radioactivity was found in the brain. At the same time, analyses carried out on the spinal cord indicate that only traces of the radioactivity could be found there.

Attempts to establish a curve for the changes in radioactivity in blood in corresponding experiments were unfortunately unsuccessful. The values obtained were altogether too irregular to permit a calculation of averages. The narcosis effect must, however, be taken into account. This much can, however, be said: within the first 10 minutes after injection, the activity in the blood is high and then rapidly falls to a value which lies below that for orthophosphate activity in brain. After 35—40 minutes,

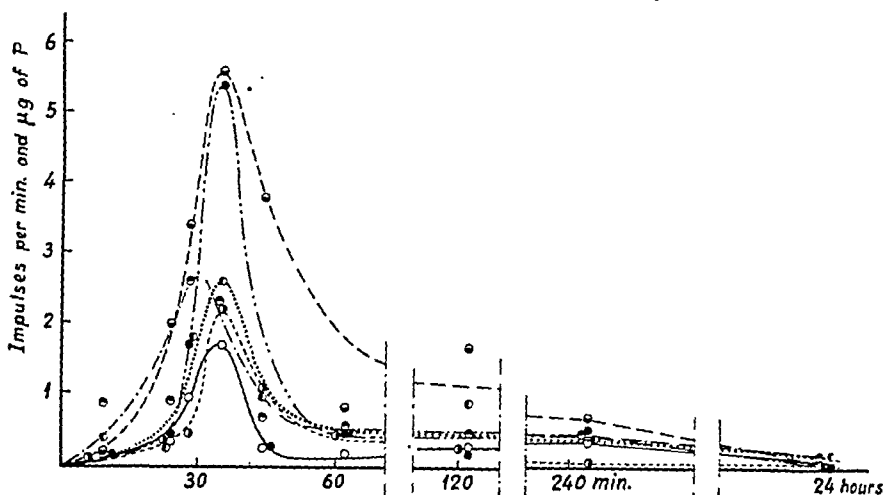


Fig. 3. Figure showing the variations of radioactivity in different portions of brain after intracerebrospinal injection of 5 microcurie radioactive sodium phosphate.

- — — — — Cortex of convexity (1 and 3)
- · - · - Subcortical layer (2 and 4)
- - - - - Corp. quadrigemina (5 and 6)
- · · · · Vermis (7)
- : - : - : Basal cortex (9 and 10)
- — — — — Central substance (11 and 12)

there is an uncertain tendency toward a maximum which later disappears.

It is clear that a part of the injected  $P^{32}$  goes from the cerebrospinal fluid directly to the blood in the first minutes after injection. Then at a later time, the phosphate activity in the blood comes from the brain. Studies in which the radioactivity was directly injected into the blood stream gave no noticeable activity in the brain, which completely supports the results of HEVESY and those of BORELL and ÖRSTRÖM (1945).

The peculiar finding that the activity is lower in the cortex than in the subcortex may be explained in full by the careful washing of the brain which not only removed the radioactivity of the cerebrospinal fluid, but also part of that in the cortex.

Differences in the time maxima for the different curves shown in Fig. 3 are not significant.

Table I shows one example of the experiments upon which the curves in Figs. 2 and 3 are based. The duration was chosen for maximal content of  $P^{32}$  in the brain. Since under no circumstances it is possible to directly estimate the metabolism of a tissue from the degree with which it takes up labelled phosphate from the

Table I.

*Radioactivity in brain after intracerebrospinal injection of  $P^{32}$ .*

Portion of brain	Weight of sample in mg	Micro g. P.	Impulses minute	Specific activity	Average spec. act.
Cortex ..... {right 3 left 1	43 52	113 123	251 245	2.2 2.0	2.1
Subcortex ..... {right 4 left 2	39 49	96 110	757 611	7.9 5.6	6.7
Corpora quadr.... {right 6 left 5	60 65	160 174	1,107 1,205	6.9 6.9	6.9
Vermis ..... 7	78	225	740	3.3	3.3
Frontal pole ..... 8	144	421	225	0.54	0.54
Cortex basal .... {right 10 left 9	51 61	130 157	400 332	3.1 2.2	2.7
Central subst. ... {right 12 left 11	48 42	166 155	— 440	— 2.8	2.8
Rest of brain	7,500	22,200	122,500	5.5	5.5

Duration of experiment = 35 minutes

Injected amount of  $P^{32}$  = 6.2 micro C

Weight of rabbit = 2.6 kg

Weight of brain = 8.17 g.

surrounding medium, the turnover of the marked phosphate which enters the cells was measured (HEVESY 1947).

The experiments in Table II show that the brain has a vigorous transphosphorylation. Since narcotics have an especially detrimental effect on these processes, and it is difficult for practical reasons to reduce the narcotic dose, a comparison of the values obtained in brain must be made with corresponding values for liver in the same animal.

### Discussion.

HEVESY and HARN (1947), HEVESY and OTTESEN (1947), believe that the amount of  $P^{32}$  which enters the brain by way of the blood circulation is very small and hence the blood circulation must play a very insignificant rôle for the uptake of phosphate



Table II.

*The turnover of radioactive phosphate in the trichloroacetic-acid soluble phosphate esters of rabbit brain and liver after injection of  $P^{32}$  into the cerebrospinal fluid.*

Sample	Inorganic phosphate <sup>1</sup> in 10 g. of tissue			Organic bound phosphate			Q
	Weight in mg	Impulses per min.	Impulses per min. and $\mu$ g. P (spec. act.)	Weight in mg	Impulses per min.	Impulses per min. and $\mu$ g. P (spec. act.)	
Brain 1	3.0	29,900	10.1	3.9	21,800	5.6	0.5
» 2	2.9	42,500	14.7	3.5	20,000	6.0	0.4
Liver 1	2.3	8,850	3.9	6.6	8,250	1.3	0.3
» 2	2.1	3,750	1.8	6.3	1,890	0.3	0.2

Weight of brain 1 = 9.98 g

» » 2 = 8.15 g

Weight of liver 1 = 127 g

» » 2 = 92 g.

Duration of experiment 1 = 38 minutes.

» » 2 = 30 »

Q = The quotient between the specific activity for organic bound phosphate and that for orthophosphate.

<sup>1</sup> Post mortem value. Cf. STONE (1943).

into the brain. We confirmed these findings when we injected  $P^{32}$  into the blood circulation. This counters the possible objection that the  $P^{32}$  which was found in the brain in the course of the spinal fluid experiments could have arrived there from the blood circulation by being rapidly resorbed into the venous sinus and then come again by way of the blood circulation.

BORELL and ÖRSTRÖM (1945) injected radioactive phosphate intraperitoneally into rats and rabbits. They found that the choroid plexus took up the radioactive isotopes 10—80 times more rapidly than the nervous tissue. In this study we found that the  $P^{32}$  which, as in their experiments, reached the nervous parts probably went over the plexus chorioideus via the cerebrospinal fluid to the brain cells. GREENBERG et al. (1943) have determined the velocity with which different ions penetrated the cerebrospinal fluid of the dog. They found that in case they employed radioactive phosphorus, an equilibrium was reached between cerebrospinal fluid and blood plasma within 2—4 hours (the authors cited their values as impulses per volume, but we recalculated them as impulses per microgram of phosphorus). It is interesting, however, that the curve for the specific activity of the orthophosphate

in the cerebrospinal fluid reached its maximum within 40—50 minutes after the intravenous injection of phosphate. Thereafter it fell off rather steeply. Throughout the entire course of an experiment, the specific activity in the cerebrospinal fluid never reached that in the blood plasma. That the curves fell off despite this can only be explained by the existence of a factor which exchanges radioactive phosphate for unmarked phosphate. This factor must be the brain.

If the results of GREENBERG et al. (1943) are compared with those of BORELL and ÖRSTRÖM (1945), one finds that the specific activity in the plexus chorioideus and the cerebrospinal fluid at a certain period is of the same order of magnitude. The barrier could therefore lie between the blood and the plexus chorioideus.

Similar relationships have been found for the ciliary body and the aqueous humour of the eye, PALM (1948).

Our experiments show that there is a rapid exchange between the cerebrospinal fluid and the brain cells measured with radioactive phosphate. From these data it follows that the need of the brain for phosphate is not as much based on the supply in the blood as rather in the spinal fluid, and that the permeability relationships hinder the uptake of blood  $P^{32}$  by the brain. If, however, its arrival to the brain is made possible by by-passing the barrier, we find that the brain incorporates radioactive phosphorus in large amounts.

It is interesting to note that the uptake of phosphate, as well as several organic dyes, into the inner secretory organs, including the vegetative centers in the brain, depends upon the blood stream. Exactly the opposite is the case with nerve tissues as demonstrated by BORELL and ÖRSTRÖM (1945). In this fundamental difference, a very important biological regulation system becomes apparent.

In our hand, it had also been possible to get a rough estimate of the capacity of the nerve cells to exchange phosphate. Despite the deep narcosis which probably has a harmful effect, a particularly vigorous phosphate turnover was observed. This recalls the observations of HYDÉN (1943), who, from studies of metabolism of the cells, concluded that the ribonucleic acid turnover in brain was very rapid, especially in the Purkinje cells of the cerebellum.

The circulation of the cerebrospinal fluid takes place from the ventricles through the fourth ventricle against the cisterna magna.

It was at this point that we injected  $P^{32}$ . From here it was distributed over the entire brain surface, and could be rapidly detected in the basal cortex and in the corpora quadrigemina and vermis. The main stream of the subarachnoidal circulation is therefore assumed to lead from the cisterna magna to the basal cisternae, cisterna ambiens, and thence to the convexity which it reaches somewhat later. It is thought, however, that irrespective of how rapidly the resorption into venous sinus may be, substances present in the cerebrospinal fluid penetrate centripetally into the brain substance to the entire depth. It is difficult to say if this could possibly take place by means of the brain veins, were this intracerebral circulation to be stopped. That it is, however, not merely a question of simple fluid flow transport follows from the results shown in Table II which demonstrate that injected radioactive phosphate is incorporated into organic compounds. In other words,  $P^{32}$  during its journey through the brain very soon comes in equilibrium with intracellular phosphate.

From the brain surface, the fluid is resorbed in the well-known manner in the venous sinus. Our studies show that one minute after injection,  $P^{32}$  can be detected in the blood, indicating a very rapid filtration. In the course of these studies we paid sufficient attention to the prevention of excess pressure in the subarachnoid space which would have accelerated resorption. WUSTMANN (1934) administered "thorotrast" in the cisterna and could detect thorium in the blood of the sinus sagittalis after two minutes.

Concerning the differences between the formation and resorption of cerebrospinal fluid, it is truly remarkable that the former, measured with  $P^{32}$  arises by a protracted secretion (50—100 minutes, GREENBERG (1943)), whereas the latter, according to our data, shows high values of  $P^{32}$  in the blood within 1—5 minutes. Similar time differences in these two processes have been established by WALLACE and BRODIE (1940) who investigated the permeability of anions.

According to MOTT's (1910) hypothesis, the cerebrospinal fluid, after having passed through the perivascular system to the depth of the brain tissue, is then resorbed in the brain capillaries. Such a centripetally oriented perivascular circulation of the spinal fluid has not, however, been detected under normal conditions (WEED (1914); SCHALTENBRAND and BAILEY (1928), WUSTMANN (1934), PATEK (1944)). Any circulation inward could be

attained only by a considerable increase in the blood osmotic pressure or by loss of blood (WEED and MC KIBBEN (1914), FOLEY (1922), PATEK (1944)).

It is nevertheless known from studies by SPATZ (1934), that dyestuffs of small molecular weight diffuse from the spinal fluid to various depths in the brain tissue. The diffusion of the dyes into the brain tissue obeyed rules similar to those of diffusion from water to a gel. The thickness of the dyed layer was related primarily to the molecular size of the dyestuff. Upon these considerations, we calculated that the resorption of the fluid and therefore of the  $P^{32}$  in the brain took place through diffusion which did not follow anatomically defined pathways. Therefore it was just those superficial regions of the brain which are richest bathed in the cerebrospinal fluid that showed the greatest activity. Accordingly, the activity of the most interior part, the corpora striata, which is furthest separated from the fluid spaces, gave a very low and more protracted curve.

The application of  $P^{32}$  gave moreover the possibility to establish that it is not only a question of simple diffusion but also a turnover of the injected  $P^{32}$  and its incorporation into organic form

### Summary.

We injected  $P^{32}$  into Cisterna magna of rabbits. The speed with which this  $P^{32}$  enters into the brain has been determined. It was found that the radioactivity increases gradually in the different parts of brain with approximately the same rapidity. The maximum was reached after 35 minutes.

The turnover of radioactive orthophosphate to organic bound phosphate in brain was measured.

The different theories for the brain protective barriers have been discussed.

The data supports the view that there are two ways of supplying the brain with  $P^{32}$ . Some minor parts mainly the hypophysis, the pineal body, the plexus chorioidei and the vegetative centers in hypothalamus are supported mainly by the blood. The nervous tissue on the other hand seems to exchange phosphate via the cerebrospinal fluid.

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From the Department of Physiology, University of Lund.

## The Chemical Transmission of Vasoconstrictor Impulses to the Hind Limbs of the Dog.

By

BJÖRN FOLKOW and BÖRJE UVNÄS.

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In previous papers (FOLKOW and UVNÄS 1948) we discussed the biological properties of the transmitter at adrenergic nerve endings in the cat. The influence of the sympatholytic drugs ergotamine and dibenamine on reflex vasoconstrictor responses was observed. Our observations indicated that the transmitter at vasoconstrictor nerve endings is not adrenaline but a substance devoid of vasodilator properties.

Some experiments were made in order to extend our observations to include the transmitter mechanism at vasoconstrictor nerve endings of the dog.

### Experimental.

The experiments were performed on dogs under chloralose-urethane anaesthesia (50 mg chloralose and 500 mg urethane per kg body weight). The animals were eviscerated leaving the liver with its arterial supply, the kidneys and the adrenals intact. The sympathetic chains were severed at the height of the second lumbar vertebra and carefully dissected free down to the fifth lumbar vertebra. The white and grey communicant fibres of this part of the chains were cut.

The blood flow was recorded separately in the caval vein, and in the saphenal veins of both legs. Two Gaddum recorders (GADDUM 1929) were used. The one received the caval outflow, the other the outflow from the vv. saphena magna et parva of both legs cannulated 2—3 cm below the knees. From the recorders the venous blood was allowed to return to the abdominal caval vein. By this arrangement the first recorder received blood predominantly from muscular, the second from cutaneous vessels of the hind legs.

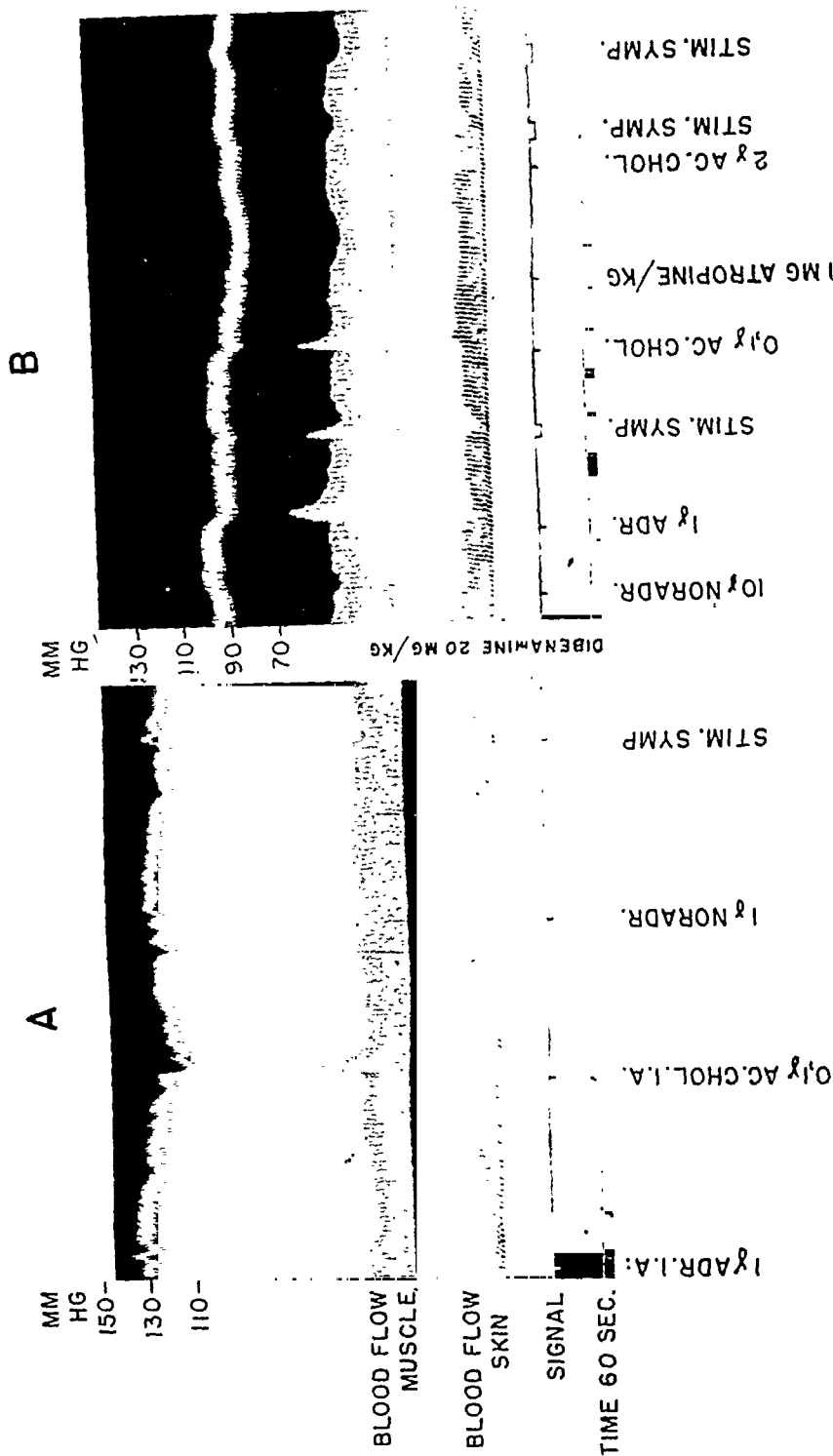
Intraarterial injections were made into the stump of the severed inferior mesenteric artery. All injections were made with a constant

volume (0.2 ml) at 37° C. As the substances were dissolved in physiological saline frequent control injections of saline were given. The blood pressure was recorded in the brachial artery by a mercury manometer. The sympathetic trunks were stimulated by a Sherrington electrode. A thyatron stimulator delivered stimuli with a frequency of 60/sec. Dibenamine or ergotamine were given slowly intravenously. Heparin was used as an anticoagulant.

## Results.

The fig. shows one of the experiments. Intraarterial injection of 1  $\gamma$  adrenaline produces after an initial reduction a moderate increase of the caval outflow. The saphenal outflow is markedly decreased. 1  $\gamma$  nor-adrenaline reduces the caval as well as the saphenal outflow. Stimulation of the sympathetic chains causes after an insignificant increase a decrease of the caval outflow. The skin blood flow is concomitantly markedly reduced. 0.1  $\gamma$  acetylcholine produces an increase of the caval as well as of the saphenal outflow. After dibenamine 20 mg/kg the picture changes. Adrenaline now exhibits a pronounced vasodilator action on the muscular vessels. The constrictor action on the cutaneous vessels is reduced. Nor-adrenaline still constricts the muscular as well as the cutaneous vessels, although the constrictor effect is markedly reduced. 10  $\gamma$  now elicits a reduction of blood flow about equivalent to the action of 1  $\gamma$  before the administration of dibenamine. The effect of stimulation of the sympathetics has changed. A pronounced increase of the caval outflow occurs, followed by a moderate decrease of the flow. The cutaneous vasoconstriction remains, although reduced. Atropine 1 mg/kg is given. This drug completely abolishes the vasodilator action of 2  $\gamma$  acetylcholine. The vasodilator effect of sympathetic stimulation also disappears and a pure vasoconstriction reappears. The vasodilator action of adrenaline remains unchanged (unfortunately not shown in figure). Identical results were obtained in experiments on six dogs. In some of these ergotamine was used instead of dibenamine.

The vasoconstrictor response to sympathetic stimulation after atropine, to nor-adrenaline, as well as to the cutaneous vasoconstrictor action of adrenaline was very resistant to the sympatholytic influence of dibenamine and ergotamine. Even after huge amounts of these drugs a weak vasoconstriction often remained. The vasoconstrictor response was never reversed to a vasodilatation.



Dog 8 kg. Chloralose-urethane. Vasomotor responses of muscular and cutaneous vessels to adrenaline, nor-adrenaline, acetylcholine and to stimulation of the abdominal sympathetic chains.

From above: Blood pressure, caval outflow, saphenal outflow, injection and stimulation marks, time.

A. Responses before dibenamine.

B. Responses after dibenamine 20 mg/kg.

Note the disappearance of vasodilator and reappearance of vasoconstrictor responses to sympathetic stimulation after atropine 1 mg/kg.



### Discussion.

Evidently dibenamine and ergotamine partially block the constrictor action of adrenaline, nor-adrenaline and vasoconstrictor impulses. Consequently the vasodilator action of adrenaline, and of vasodilator impulses is revealed. Since the sympathetic vasodilators to the muscles of the hind legs of the dog are cholinergic (BÜLBRING and BURN 1935) the vasodilator responses to stimulation of the sympathetic chains are blocked by atropine. *The incompletely blocked vasoconstrictor action then reappears.* Since there is no indication that dibenamine or ergotamine interferes with the release of the adrenergic transmitter, this observation strongly indicates that the transmitter at vasoconstrictor nerve endings lacks dilator action on the muscular vessels. In our view this fact excludes adrenaline as the transmitter. To comply with our observations the transmitter should be a potent sympathomimetic principle devoid of vasodilator action. Nor-adrenaline fulfils this requirement (EULER 1946, FOLKOW et al. 1948).

Our present observations on dogs are in full agreement with our previous observations on cats that the transmitter at vasoconstrictor nerve endings is a substance lacking vasodilator action. The transmitter might be nor-adrenaline or a substance with similar biological properties.

The action of adrenaline, nor-adrenaline and stimulation of the sympathetics on the cutaneous vessels will be discussed in a later paper.

### Summary.

The transmitter of sympathetic vasoconstrictor impulses to the hind limbs of the dog lacks vasodilator action. The transmitter is not adrenaline. It might be nor-adrenaline or a substance with similar biological properties.

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# The Sympathetic Vasomotor Innervation of the Skin of the Dog.

By

BJÖRN FOLKOW, JØRGEN FROST, KNUT HÆGER  
and BÖRJE UVNÄS.

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In a series of papers BÜLBRING and BURN (1935, 1936, 1937) investigated the distribution and functional significance, of sympathetic vasodilator nerves in different animal species. No sympathetic vasodilator supply was found to the skin, except to the skin of the ear of the dog. According to these authors the sympathetic vasodilator fibres are of two types, cholinergic and adrenergic. Since BÜLBRING and BURN were unable to detect any dilator action of acetylcholine on cutaneous vessels of the dog they concluded that the sympathetic vasodilator fibres to the ear were not cholinergic.

In a series of papers we argue that sympathetic vasodilator fibres generally might be cholinergic (FOLKOW et al. 1948). Since we (FOLKOW and UVNÄS 1948) unlike BÜLBRING and BURN observed a pronounced dilator action of acetylcholine on cutaneous vessels, the following experiments were performed.

## Experimental.

The experiments were performed on dogs under nembutal (30 mg/kg) or combined chloralose (50 mg/kg)-urethane (500 mg/kg) anaesthesia. Observations were made on the cutaneous vessels of the hind limbs and of the ear. Adrenaline, nor-adrenaline and acetylcholine were given intraarterially, dibenamine and ergotamine slowly intravenously. For nerve stimulation a Sherrington electrode and a thyatron stimulator delivering 60 impulses per second were used.<sup>1</sup>

<sup>1</sup> The blood pressure was measured in the brachial artery by a mercury manometer. Heparin was used as an anticoagulant.

### Experiments on Hind Limbs.

In four experiments the experimental arrangement was the same as described by FOLKOW and UVNÄS (1948). The caval and the combined saphenous outflows from both hind limbs were registered separately by two Gaddum recorders. In five other experiments the cutaneous flow was registered in the following way. The one femoral artery was ligated just distally to the origin of the saphenous artery. A branch of the femoral artery just proximally to this point was isolated and cannulated for "close arterial injections" to the saphenous vascular region. The venous outflow from both saphenous veins of the legs was registered by an electrical drop recorder connected to an "Ordinatenschreiber". In these tracings the height of the record measures the interval between successive drops.

#### *Results.*

Experiments were performed on 9 dogs. Stimulation of the abdominal sympathetic chains produced a marked cutaneous vasoconstriction. This constrictor effect was considerably reduced but never annulled by the sympatholytic effect of dibenamine or ergotamine, not even if these drugs were given concomitantly in huge doses (dibenamine 30 mg and ergotamine 5 mg per kg body weight). The vasoconstrictor effect was never observed to be reversed to a vasodilator effect under the influence of these drugs.

Intraarterial injections of adrenaline and of nor-adrenaline produced a vasoconstriction of about the same magnitude (see fig. 1). No vasodilator action was observed by small doses of these drugs. The vasoconstrictor effects were reduced but usually not abolished by dibenamine or ergotamine. Under the influence of huge doses of these drugs adrenaline occasionally produced a slight vasodilatation. In all experiments acetylcholine proved to have a pronounced vasodilator action on the cutaneous as well as on the muscular vessels.

The action of adrenaline, nor-adrenaline and acetylcholine on the saphenal outflow is shown in fig. 1. Identical results were obtained in all 9 experiments.

### Experiments on the Skin of the Ear.

The one common carotid was isolated and all branches were ligated except those supplying the ear. Intraarterial injections were made into the cannulated stump of the lingual artery. The vago-sympathetic

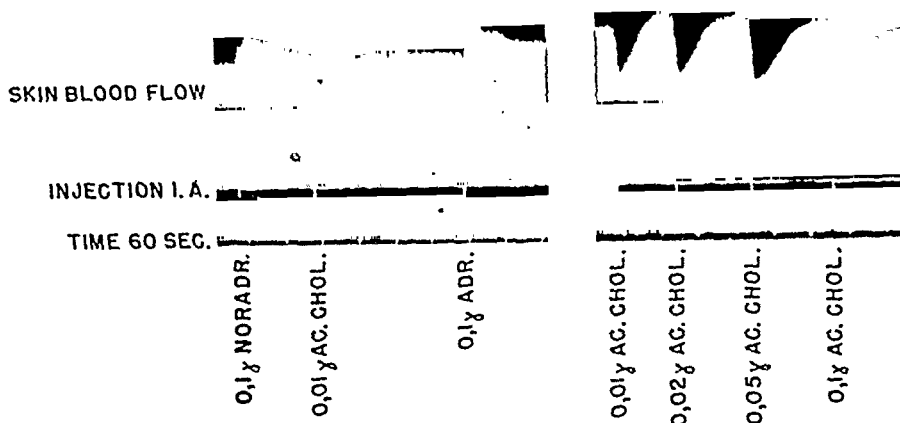


Fig. 1. Dog 6 kg. Chloralose-urethane. Blood pressure 130 mm Hg. Saphenal outflow of right leg. Action of adrenaline, nor-adrenaline and acetylcholine given intraarterially.

trunk was isolated in the neck and cut a few centimeters above the thorax aperture. In order to exclude reflex vascular responses in the ear due to afferent vagal impulses the vagus was severed just where it emerges from the skull. Care was taken to leave the sympathetic fibres intact. The two marginal veins of the ear were cannulated and the venous outflow registered by an electrical drop recorder connected to an "Ordinatenschreiber" as mentioned above.

### Results.

Experiments were carried out on 12 dogs.

Stimulation of the sympathetic trunk produced a very pronounced vasoconstriction in the ear (see fig. 2). We were not able to block this constrictor effect completely even if huge doses of dibenamine and ergotamine were given. Adrenaline and nor-adrenaline elicited vasoconstrictions of about equal magnitude. No vasodilatation with small doses was ever observed. The constrictor action of nor-adrenaline was more or less completely blocked by ergotamine or dibenamine but the constriction was never reversed to a dilatation. Occasionally the constrictor action of adrenaline changed into a slight dilator action under the influence of large doses of dibenamine and ergotamine (see fig. 2). As was the case with the skin of the hind limbs intraarterial injection of acetylcholine produced a pronounced vasodilatation in the ear.

The actions of adrenaline, nor-adrenaline and acetylcholine on the skin of the ear are shown in fig. 2 and 3.

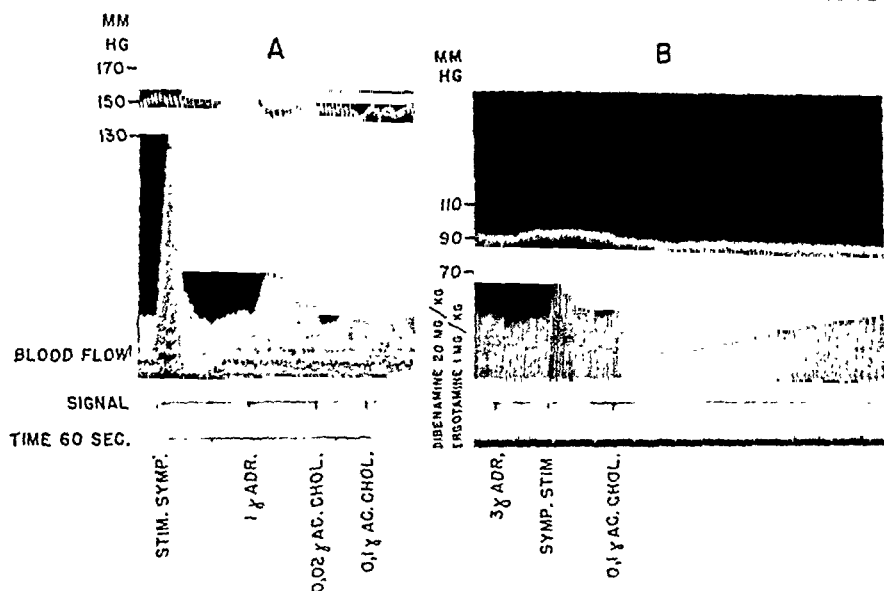


Fig. 2. Dog 12 kg. Chloralose-urethane. Venous outflow from the marginal veins of the ear registered by "Ordinatenschreiber".

A. Vasoconstrictor action of stimulation of the sympathetic trunk in the neck, and of adrenaline. Vasodilator action of acetylcholine.

B. The influence of dibenamine and ergotamine on the vasomotor responses to stimulation of the sympathetics, to adrenaline and to acetylcholine.

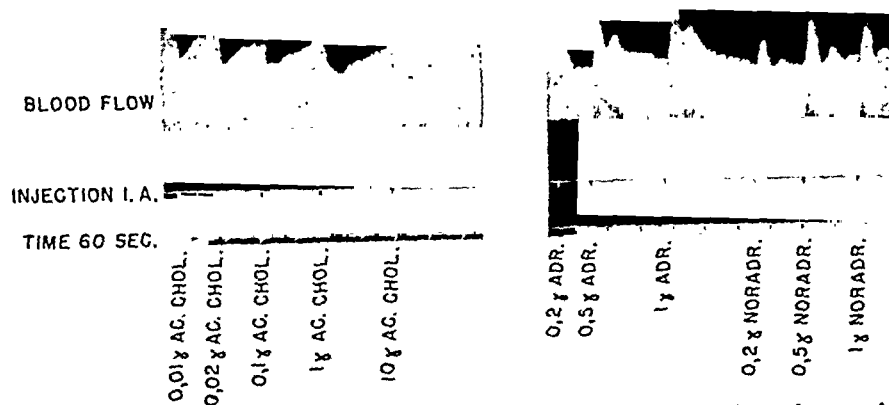


Fig. 3. Dog 7.5 kg. Nembutal. Blood pressure 110 mm Hg. Outflow from the marginal veins of the ear registered by "Ordinatenschreiber". Action of acetylcholine, adrenaline and nor-adrenaline given intraarterially.

## Discussion.

BÜLBRING and BURN (1936) claim the existence of sympathetic vasodilator fibres to the skin of the ear of the dog. Under the influence of ergotamine stimulation of the sympathetic trunk in the

neck was observed to produce an increase of the volume of the ear. This increase of volume was unaffected by atropine, or by eserine. Further BÜLBRING and BURN were unable to demonstrate any dilator action of acetylcholine on cutaneous vessels. Consequently they concluded that the supposed sympathetic vasodilator nerves to the skin of the ear were not cholinergic.

Our observations do not agree with those of BÜLBRING and BURN. We have not been able to find any evidence for the existence of sympathetic vasodilators to the skin of the ear of the dog. The vasoconstriction produced by stimulation of the sympathetics in the neck was never reversed to a vasodilatation under the influence of the sympatholytic drugs dibenamine and ergotamine. We are not convinced that the results obtained by the use of the plethysmographic technique of BÜLBRING and BURN justify the conclusions made by them. Even used in skilled hands this indirect recording technique has its disadvantages. Further, when covering the ear of the dog the plethysmograph will enclose the muscles of the ear as well. The records will then include the vasomotor reactions in these muscles.

Our observations that the skin of the dog lacks sympathetic vasodilator innervation agrees with our previous observations on the cutaneous sympathetic vasomotor innervation of the cat.

Against the claims of BÜLBRING and BURN we have observed acetylcholine to have a pronounced dilator action on cutaneous vessels. In our view acetylcholine is well fitted to be the transmitter at nerve endings of cutaneous vasodilator nerves.

Adrenaline and nor-adrenaline exert a pronounced cutaneous vasoconstriction of about equal magnitude. This observation is in agreement with our claim (FOLKOW et al. 1948) that nor-adrenaline might be the transmitter at vasoconstrictor nerve endings.

### Summary.

No evidence was found for the existence of a sympathetic vasodilator innervation to the skin of the hind limbs or the ear of the dog.

Nor-adrenaline has a pronounced vasoconstrictor action on cutaneous vessels. The observation agrees with the idea that the

substance might be the transmitter at vasoconstrictor nerve endings.

Adrenaline has a pure vasoconstrictor action on cutaneous vessels, even in low concentrations.

Acetylcholine has a pronounced vasodilator action on cutaneous vessels. Thus it cannot be excluded as a possible transmitter at cutaneous vasodilator nerve endings.

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## **Action of Acetylcholine, Adrenaline and Noradrenaline on the Coronary Blood Flow of the Dog.**

By

BJÖRN FOLKOW, JØRGEN FROST and BÖRJE UVNÄS.

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Acetylcholine dilates the coronaries of the turtle and the rabbit (SMITH et al. 1926), of the cat (WIGGERS 1909) and of the dog (KATZ et al. 1938). On the coronaries of the cat KATZ et al. in some experiments observed a dilatation, in others a constriction when acetylcholine was injected. Recently ECKENHOFF et al. (1947) reported that acetylcholine in minute doses (less than  $1\gamma$ ) dilates the coronaries of the dog.

WIGGERS (1909) and RABE (1912) observed a constrictor action of adrenaline on the coronaries of the cat. SMITH et al. claim that adrenaline in low concentrations reduces the coronary blood flow in the turtle and the rabbit. In higher concentrations adrenaline increased the flow. Concomitantly the activity of the heart muscle increased. KATZ et al. (1938) and recently ECKENHOFF et al. reported an increased coronary flow when adrenaline was given intraarterially.

FOLKOW et al. (1948 a) observed the appearance of a substance with the biological properties of acetylcholine in the coronary perfusate of the dog and cat when the stellate ganglia were stimulated. The observations were taken to indicate the existence of cholinergic dilator fibres in the sympathetic outflow to the coronaries. Since this conception is opposed to the generally accepted idea that the sympathetic coronary dilator fibres are adrenergic, we considered it desirable to reinvestigate the action of acetylcholine and adrenaline on the coronaries of the dog. As nor-



adrenaline has no vasodilator action on the muscular, cutaneous and splanchnic vessels of the cat and dog (Folkow et al. 1948 b) the action of this substance on the coronaries was studied as well.

## Experimental.

The experiments were performed on dogs under nembutal anaesthesia (30 mg/kg i. v.). Cross-circulation was arranged between two dogs. The coronaries of a heart in situ were perfused with blood from a donor dog. After the cross-circulation had been established the recipient animal was allowed to die. In this way nervous influences on the perfused coronaries were excluded. Further the cross-circulation arrangement had the advantage of giving a perfusion pressure that was uninfluenced by the substances injected into the coronaries. The minute amounts of the drugs sufficient to produce a pronounced action on the coronaries were without any noticeable effect on the vascular system of the donor animal.

Cross-circulation was arranged as follows.

*Recipient.* Under artificial respiration the anterior wall of the chest was removed. The aortic arch with its large ascending branches, the upper and lower caval veins and the vena azygos were ligated. Both the hili of the lungs were ligated and the right lung removed. The pericardial sac was opened, the two main coronary arteries prepared free at their origin from the aorta and cannulated. The coronary outflow was collected through a Morawitz cannula inserted into the coronary sinus.

*Donor.* The carotid artery and the jugular vein were prepared free in the neck, ligated and cannulated. To prevent coagulation 0.1 mg/kg heparin was given.

The perfusion was then started. The coronaries received their blood from the carotid artery of the donor. The outflow from the coronary sinus was directed to a Gaddum recorder (GADDUM 1929) and the blood then returned to the jugular vein of the donor. To prevent the accumulation of blood in the heart cavities both ventricles were drained by cannulas pushed into them through the muscular wall.

The blood pressure of the donor was recorded in the brachial artery by a mercury manometer. "Close arterial injections" were made into the rubber tube connected to the coronary cannulas. Each injection was standardized to 0.2 ml total solution kept at 37° C. As dilutions were made with physiological saline frequent control injections with plain saline were given. Saline was without any noticeable effect on the coronary outflow.

In our perfusion arrangement the heart fibrillated spontaneously in three experiments. In the other four experiments the rhythmic heart activity persisted but the heart muscle could easily be thrown into fibrillation by strong electrical stimulation of the right auricle. We preferred to experiment on the fibrillating heart in order to minimize the variations of heart muscle activity.



Cross-circulation. Donor 8 kg. Recipient 6 kg. Nembutal 30 mg/kg. Action of acetylcholine, adrenaline and nor-adrenaline on coronary blood flow.

### Results.

In all experiments intraarterial injections of acetylcholine in amounts from 0.01% and upward produced a marked increase of the coronary outflow (see fig.). Adrenaline, and nor-adrenaline increased the coronary outflow as well. The increase was less pronounced but more protracted than that produced by acetylcholine. Nor-adrenaline was the less potent of the two sympathomimetic substances. The amounts of adrenaline and nor-adrenaline necessary to produce an increase of the coronary outflow concomitantly increased the activity of the fibrillating as well as of the rhythmically beating heart muscle.

### Discussion.

According to current teaching coronary dilator fibres run in the sympathetics, mainly via the stellate ganglia (for references see FOLKOW et al. 1948 a). The observations that acetylcholine is a very potent coronary dilator substance are in agreement with the idea recently brought forward (FOLKOW et al.) that this substance is the transmitter at sympathetic coronary dilator fibres, at least in the dog.

Adrenaline, and to a minor degree nor-adrenaline were observed to produce an increase of coronary blood flow. In our view these

observations do not justify the conclusion that the coronary dilator fibres are adrenergic. A promoting action of adrenaline on heart muscle activity and coronary blood flow is in agreement with the emergency theory of adrenal function as originally presented by CANNON. According to this theory the function of adrenaline is to accentuate and enhance the redistribution of blood from relatively inactive to active regions of the body. Adrenaline as well as nor-adrenaline increase the coronary blood flow only when given in amounts that concomitantly stimulate the heart. It remains to be shown to which degree the increase of the coronary flow produced by these substances is not a consequence of the increased activity of the heart muscle. As regards nor-adrenaline this substance seems to lack vasodilator properties on other vascular regions (EULER, FOLKOW et al.). A direct dilator action on the coronary vessels should form the only exception so far observed.

The chemical nature of the adrenergic transmitter is still under discussion. Recent observations by EULER (1945), and by FOLKOW and UVNÄS (1948) indicate that nor-adrenaline or a substance with similar biological properties might be the mediator substance. In our view the observation that nor-adrenaline on intraarterial injection produces a minor increase of the coronary blood flow does not invalidate the conception that nor-adrenaline or a similar substance might be the transmitter at the coronary constrictor nerve endings. Physiologically the transmitter is released at the nerve endings close to the receptor cells thus permitting a strictly local action of the released substance on the arteriolar muscle. Under our experimental conditions nor-adrenaline was given intraarterially thus distributed not only to the coronary arterioles but to the heart muscle as well. The slight increase of the coronary flow produced by this substance might well be secondary to the concomitant increase of the activity of the heart muscle.

### Summary.

Acetylcholine has a pronounced dilator action on the coronaries of the dog. The observation is in agreement with our previous suggestion that the sympathetic coronary dilator fibres in the dog are cholinergic.

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From the Department of Physiology, University of Lund.

## The Gastric Secretory Response to Insulin in Dogs and Cats.

By

PRITS JÖGI and BÖRJE UVNÄS

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After some years of controversial reports (MEYER 1930, OKADA et al. 1929, LA BARRE and CESPÉDÈS 1931, KALK and MEYER 1932, DOBREFF 1931—32, BOLDYREFF and STEWART 1932 and others), it is now generally assumed that the gastric secretory response to insulin is due to hypoglycemia.

During the course of investigations concerning CNS regions the activation of which induces a gastric secretory response to insulin, we made some observations that might be of interest to investigators in this field.

### Experiments on Dogs.

#### Method.

Gastric fistula dogs were used. They were starved for 20 hours before the experiment. After a control period of an hour insulin was given intravenously. The volume of the secretion was measured in 15 minute periods and the samples were titrated colorimetrically for free and total acidity against N/10 NaOH. Di-methyl-azo-benzol and phenolphthalein were used as indicators. Blood was drawn from the ear vein every 15 minutes and blood sugar determined according to the micromethod of FOLIN (1928), slightly modified to suit the Coleman Junior Spectrophotometer.

#### Results.

Confirmatory to earlier reports a gastric secretory response to insulin occurred when the hypoglycemia reached values

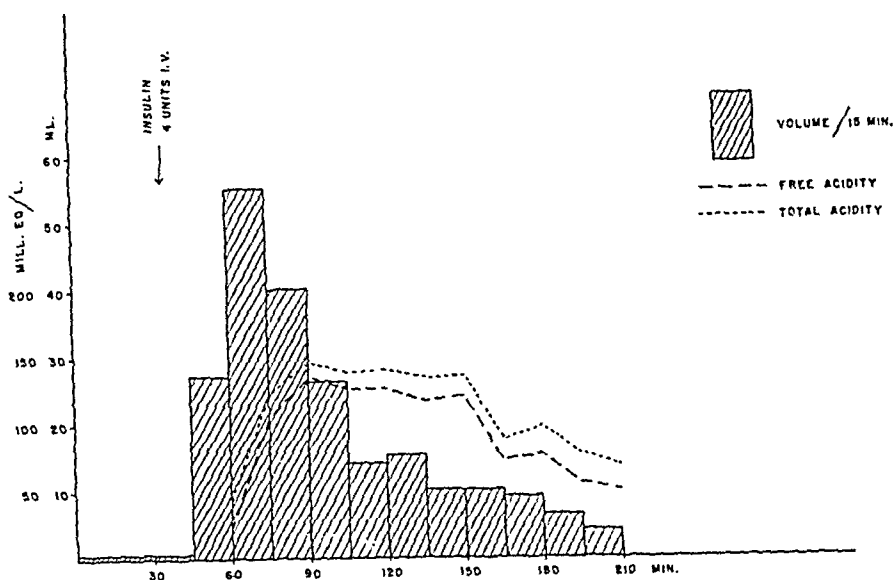


Fig. 1. Dog 20 kg. Gastric secretory response to a moderate dose of insulin (0.2 units per kg).

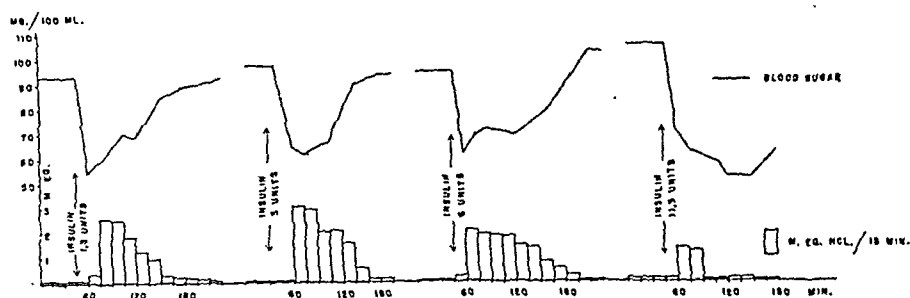
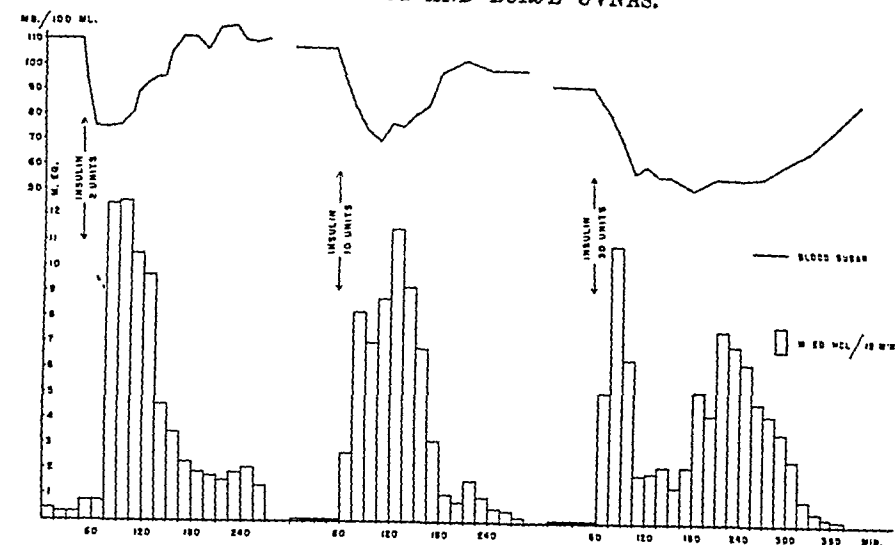


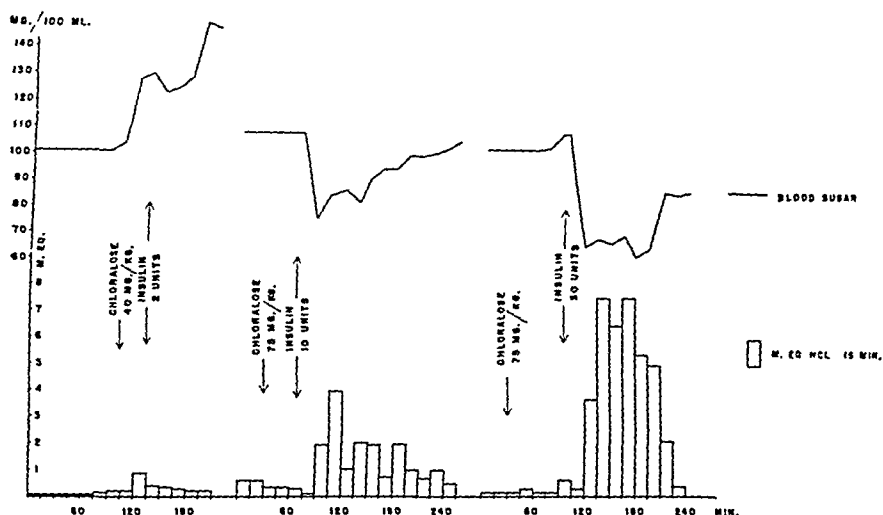
Fig. 2. Dog 10 kg. Gastric secretory response (m. eq. HCl) to various doses of insulin.

between 60–80 mg per cent. The secretion started 10–15 minutes after insulin was given. When moderate doses of insulin were used the secretion reached a peak in 30–45 minutes and then gradually declined to the basic level in 1–2 hours. The acidity as well as the peptic activity of the juice was high. A typical secretory response to hypoglycemia is seen in fig. 1.

Inducing hypoglycemia with insulin is a very reliable way to activate the gastric glands. Out of 40 dogs observed by us during the past years only one has not responded to insulin. However, to get a good secretory response the dose of insulin has to be carefully selected. The sensitivity of the dogs to insulin varied considerably and in many animals the gastric secretion was in-



a.



b.

Fig. 3 a and b. Dog 28 kg. Influence of chloralose on gastric secretory response to insulin.

hibited if the doses of insulin given were too high. Usually 0.1—0.2 units of insulin per kg body weight induced an optimal secretory response. When doses of 0.5—1.0 units per kg were given the secretory response frequently declined considerably or completely disappeared. The secretory responses of a dog to various doses of insulin are presented in fig. 2.

For our investigations it was important to know the effect of anaesthesia on the secretory response to insulin. 5 dogs were

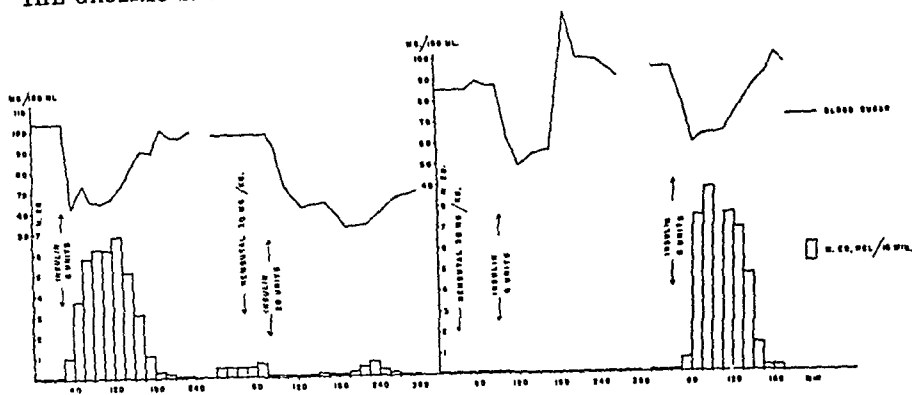


Fig. 4. Dog 18 kg. Influence of nembutal (30 mg/kg) on gastric secretory response to insulin.

selected for an investigation on this subject. The secretory response to different doses of insulin was determined. Interrupted by frequent control experiments the dogs were then anaesthetized once a week and the secretory response to insulin recorded. The anaesthetics subjected to investigation were chloralose, nembutal (pentobarbital), ether and morphine.

*Chloralose.* Chloralose was given intravenously in hypnotic doses (60—75 mg/kg). As seen in fig. 3 a and b, in these doses chloralose completely abolishes the secretory response to moderate doses of insulin. Higher insulin doses still elicit a secretion although this is markedly reduced.

Chloralose itself induced a gastric secretion. It is a well known experience that chloralose, especially when given in inadequate hypnotic doses, causes excitatory phenomena of central nervous origin. It was a frequent observation that under such conditions the gastric glands were activated. A highly acid gastric juice was secreted. This secretion was sometimes so copious that it prevented the performance of the actual experiment.

*Nembutal.* As a representative for the barbiturate group nembutal was chosen. This substance induces a short but reliable anaesthesia. As seen in fig. 4 nembutal completely prevented the secretory response to insulin. Although the blood sugar was depressed, even high doses of insulin were ineffective. On the other hand the secretory response to histamine was not significantly depressed by the doses of nembutal used.

*Ether and morphine.* Both drugs caused a pronounced hyperglycemia that was not depressed by insulin in doses up to 1 unit per kg. Consequently no gastric secretion occurred.



It was of interest to us to know how soon the secretory response to hypoglycemia reappeared after the end of the anaesthesia. We found that regardless of which anaesthetic used the secretory responses became normal as soon as the animal recovered consciousness.

### Comments.

LA BARRE and CESPÉDÈS (1931) claimed that the secretory response to insulin was not depressed by chloralose. According to our findings the secretion is markedly depressed by this anaesthetic although a secretory response still remains. The secretagogue effect of chloralose itself is a disadvantage that necessitates a careful interpretation of results obtained when this anaesthetic is used.

Contrary to chloralose nembutal completely prevents the secretory response to insulin. As the blood sugar is depressed and histamine as usual elicits gastric secretion the inhibitory effect of nembutal is probably due to a depressive action on the cerebral structures activated by the hypoglycemia.

The inability of insulin to induce gastric secretion under ether and morphine obviously is due to the fact that these drugs cause a hyperglycemia that is not depressed by the insulin doses used in our experiments.

### Experiments on Cats.

Numerous attempts were made on cats, anaesthetized as well as unanaesthetized, to induce a gastric secretory response to insulin. In spite of the occurrence of a hypoglycemia, that according to our experiences from dogs should evoke a gastric secretion, we were never able to activate the gastric glands. According to GELLHORN (1938), hypoglycemia stimulates sympathetic as well as parasympathetic regions in the central nervous system. It might be, that in the cat hypoglycemia predominantly excites sympathetic structures.

### Summary.

Nembutal, ether and morphine in hypnotic doses completely abolish the gastric secretory response to insulin. Chloralose depresses but does not prevent the secretory response. The secre-

tory responses become practically normal as soon as the animals regain consciousness.

Insulin hypoglycemia does not evoke any gastric secretion in cats.

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From the Department of Physiology, University of Lund.

## The Origin in the CNS of Gastric Secretory Impulses Induced by Hypoglycemia.

By

PRITS JÖGI, GUNNAR STRÖM and BÖRJE UVNÄS.

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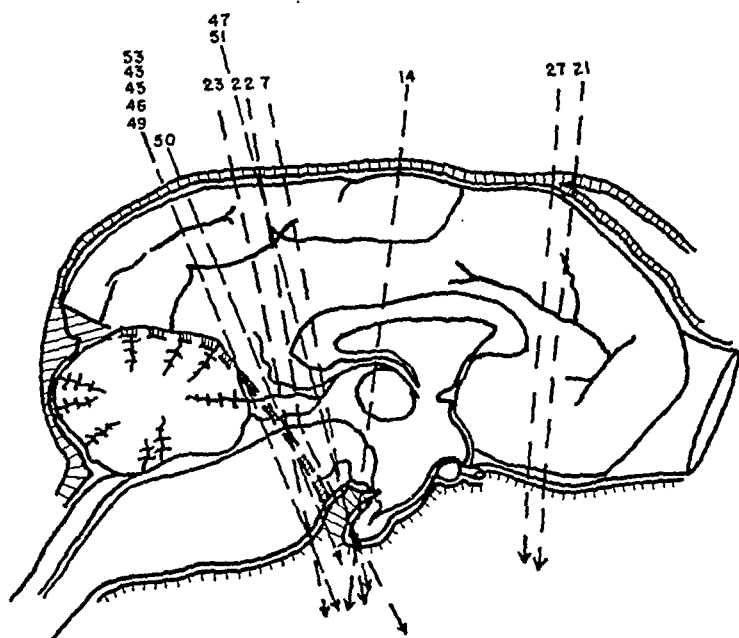
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Hypoglycemia produced by the intravenous injection of insulin is able to induce the discharge of vagal secretory impulses to the gastric mucosa of dog and man. LA BARRE and CESPÉDÈS (1931) consider the gastric secretory response to be due to the activation by the hypoglycemia of "vagal centres" of the brain. In cross-circulation experiments they observed a secretion of HCl in the stomach of a recipient animal when the head of this animal was perfused with hypoglycemic blood from a donor dog.

The recent favourable reports on the healing of chronic peptic ulcer in man after bilateral vagotomy has increased the interest in the problems concerning the influence of the CNS on the secretory activity of the stomach. The present experiments were devoted to an investigation on the origin in the CNS of the vagal gastric secretory impulses induced by hypoglycemia.

### Experimental.

The experiments were performed on gastric fistula dogs. The gastric secretory responses to various doses of insulin were determined once or twice a week. Before an assay the dogs were starved for 20 hours. Insulin was given intravenously. The volume of the gastric secretion was measured every 15 minutes and the amount of HCl secreted determined by colorimetric titration (N/10 NaOH and phenolphthalein). In all experiments the blood sugar level was followed by the determination of the blood sugar concentration every 15 minutes.



WITH ITS BONY WALLS.

Fig. 1. Schematic drawing illustrating the height of the frontal sections through the brains. Sagittal midsection.

Blood was taken from the vein of the ear and blood sugar determined by the micro method of FOLIN (1928) adapted for the Coleman Junior spectrophotometer.

The secretory responses to various amounts of insulin established, the dogs were subjected to various brain operations. As previously reported by JÖGI and UVNÄS (1948) nembutal (pentobarbital) completely abolishes and chloralose markedly reduces the gastric secretory response to insulin. Consequently we had to desist from making our observations in acute experiments. The dogs were operated on under sterile conditions, nembutal being used as the anesthetic. The secretory response to insulin was determined the day after the operation and then regularly once or twice a week as long as the animal stayed alive in good condition. In cases where hypoglycemia did not induce any gastric secretion the secretory ability of the parietal cells was controlled with histamine 0.5 mg subcutaneously as the secretory stimulant.

The operative procedures were as follows.

A. *Decortication.* Five dogs were decorticated. The operation was performed in two stages. In the first stage the skull was widely opened on one side and the cortex of this side removed by suction according to the technique used by BARD and others. The animal was allowed to recover for 2—3 weeks. The decortication was then completed by removal of the cortex of the other side.

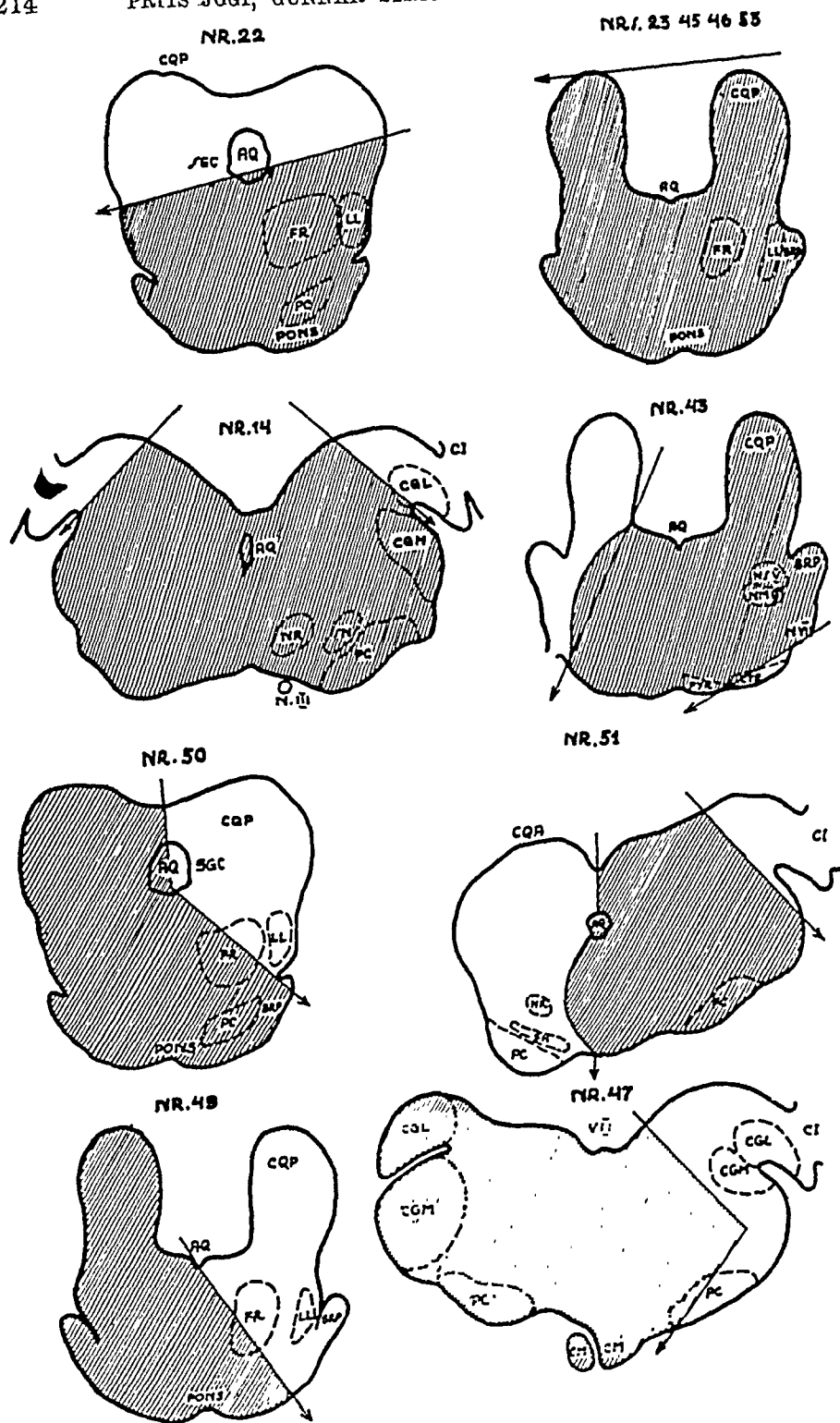


Fig. 2.

B. *Frontal sections of cortex and brain stem.* Cross sections were made at various heights in 12 dogs. As appears from fig. 1, 10 of the dogs might be considered as decerebrated. The sections were made by two different techniques.

a) On three dogs one parietal lobe was partially removed and the exposed brain stem sectioned by the suction technique (No. 14, 22 and 23 in fig. 1). The other dogs were decerebrated according to the technique described by KELLER (1943). The skull was opened over the left occipital lobe, which was pushed aside to expose the brain stem. This was then severed by a blunt-ended instrument.

All animals were given penicillin prophylactically, 5,000 units every fifth hour the first three postoperative days. If necessary the animals were nutreated through the gastric fistula. The body temperature of the decerebrate animals was controlled every fifth hour and kept at 37–40° C by adjusting the temperature of the surroundings to an appropriate level. These precautions taken we were able to keep the decerebrate animals alive in a satisfactory condition up to 50 days. Post mortem the brains were removed, fixed in formaline and the result of the operations examined in macroscopical sections (fig. 1 and 2).

## Results.

The gastric secretory responses to hypoglycemia before and after the brain operations appear in the table.

*Decorticate animals.* After decortication a temporary hyperthermia and hyperglycemia appeared in the dogs. During the 3 weeks following the operation the body temperature amounted to 38.5–39° C. Otherwise the general condition of the animals was good. Their behaviour was typical for decorticate animals with occasional excitatory outbursts. These excitatory manifestations were especially apt to occur when the animals were used in an experiment. During the first few days following the decortication the secretory response to insulin disappeared or was markedly

Fig. 2. Schematic drawings illustrating the extension of the decerebrate sections.

Abbreviations used:

AQ = aqueductus Sylv.  
BRP = brachium pont.  
CGL = corpus geniculat. lat.  
CGM = corpus geniculat. med.  
CI = capsula int.  
CM = corpus mamill.  
CQA = corpus quadrig. ant.  
CQP = corpus quadrig. post.  
CTR = corpus trapezoid.

FR = formatio reticul.  
LL = lemniscus lat.  
NR = nucleus ruber  
NM V = nucl. motor. nerv. trigemin.  
NS V = nucl. sens. nerv. trigemin.  
PC = pedunculus cerebr.  
PYR = tractus corticospin.  
SGC = substantia gris. centr.  
SN = substantia nigra

Table.

Dog nr	Operation	day after ope- ration	Before insulin		dose of insulin IU/kg	After insulin			Hista- mine control m. eq. HCl, 0—60 min.
			blood sugar mg%	m. eq. HCl, 60 min.		blood sugar mg%	m. eq. HCl, 0—60 min.	m. eq. HCl, 60—120 min.	
25	Decortic. dx.	preop.	94	5.58	0.26	43	10.97	5.95	
		2	119	2.28	0.26	63	1.40	1.97	
		7	93	0	0.26	58	0.50	2.35	
		16	82	2.52	0.26	49	3.58	6.22	
	Decortic. sin.	2	128	4.84	0.26	78	2.88	0.72	20.0
		9	138	1.04	0.26	65	4.78	1.11	
		15	100	2.22	0.26	49	1.65	2.16	
		23	95	2.44	0.26	51	1.57	1.97	
		31	96	1.24	0.15	55	5.34	8.49	6.96
		40	105	0.36	0.07	78	5.40	3.83	
		58	103	4.88	0.15	61	4.35	4.56	
		101	84	2.42	0.15	49	1.77	0.53	
		107	91	0.20	0.11	53	4.49	2.19	
27	Frontal lobe section	1	97	0.12	0.25	40	0.94	4.53	6.91
		10	85	0	0.25	44	4.19	5.04	
	Decortic. dx.	4	92	0.16	0.25	39	1.00	4.09	
		14	101	0.68	0.25	43	4.36	4.77	
	Decortic. sin.	1	110	1.76	0.25	38	2.29	3.13	
		5	112	1.32	0.25	58	1.51	1.00	
		12	109	0.76	0.25	50	1.87	2.16	
		19	100	0.48	0.25	46	2.26	2.99	
36	Decortic.	preop.	89	0	0.16	58	8.08	6.48	
		19	88	0	0.16	54	3.89	4.57	
37	Decortic.	preop.	91	0.76	0.16	56	6.38	8.49	
		26	92	1.00	0.16	52	1.00	0.73	
39	Decortic.	preop.	100	0	0.15	59	2.88	0.46	
		9	100	0.60	0.125	46	2.97	3.74	
21	Frontal lobe section	1	121	3.52	0.50	60	2.72	2.12	
		4	99	1.10	0.25	64	5.87	8.09	
		9	101	1.98	0.25	48	3.29	5.27	
14	Decerebr.	preop.	86	1.12	0.25	50	6.04	14.90	
		1	120	0.64	0.33	52	0.22	3.54	
		4	94	1.06	0.25	43	0.54	2.33	
22	Decerebr.	preop.	75	0.72	0.25	51	3.05	1.04	13.11
		1	101	0.16	0.25	56	0.20	0.20	
		7	99	1.90	0.25	79	0.80	0.40	
		10	82	0	0.50	45	0	0.30	
23	Decerebr.	preop.	73	1.60	0.25	50	6.64	5.00	7.83
		1	122	0	0.25	74	0.40	0.80	
		3	90	0.88	0.25	56	0.60	5.99	

Table. (Cont.)

Dog nr	Operation	day after operation	Before insulin		dose of insulin IU/kg	After insulin			Hista- mine control m. eq. HCl, 0—60 min.
			blood sugar mg%	m. eq. HCl 60 min.		blood sugar mg%	m. eq. HCl, 0—60 min.	m. eq. HCl, 60—120 min.	
43	Decerebr.	preop.	78	2.24	0.17	44	6.40	3.10	5.93
		1	101	0	0.17	59	0	0	
		4	105	0	0.25	63	0	0	
		6	115	0	0.25	59	0	0	
		12	104	0.12	0.42	53	0.08	0.67	
		19	102	0	0.33	63	0	0.19	
		26	94	0.80	0.21	63	0.16	0.16	
		41	140	0.24	0.92	61	0.55	5.80	
		44	110	0.60	0.17	56	0.20	4.20	
		48	109	0	0.17	59	0	1.36	
45	Decerebr.	preop.	90	0	0.10	51	3.17	2.07	6.41
		1	84	0.44	0.10	71	0.60	0.30	
		5	82	0	0.20	58	0	0	
		8	101	0	0.33	69	0	0	
		12	100	0	0.40	64	0	0	
		22	91	0	0.54	56	0	0.28	
46	Decerebr.	preop.	99	0	0.12	62	4.24	7.10	1.78
		1	99	0.40	0.23	63	0.14	0	
		5	109	0.60	0.30	80	0.28	0.18	
47	Decerebr.	preop.	105	0	0.23	60	3.22	7.54	1.81
		1	103	0	0.23	81	0	0	
49	Decerebr.	preop.	95	0	0.36	69	3.47	4.16	7.76
		1	111	0.44	0.36	71	0.80	1.57	
		7	108	1.30	0.21	71	0.75	1.27	
		13	92	1.48	0.36	71	0.76	0.91	
		18	95	2.06	0.86	72	0.74	0.32	
		22	100	0.66	0.43	68	0.26	0.27	
		25	104	1.40	0.36	62	3.18	4.48	
		27	97	0	0.21	68	0.14	0.28	
50	Decerebr.	preop.	80	0	0.36	59	3.17	7.59	5.16
		1	108	0	0.36	62	0	0	
51	Decerebr.	preop.	117	1.62	0.19	69	6.85	7.33	5.16
		1	115	5.60	0.19	75	5.11	5.51	
		10	109	0.54	0.19	65	0.65	0.60	
		12	108	1.50	0.50	61	1.47	1.72	
		17	111	1.00	1.00	61	1.62	1.04	
		19	110	0	0.31	80	0	0.09	

reduced. The response then gradually increased during the following 2—3 weeks but usually remained moderately reduced. The decortication did not change the character of the gastric secretory



response. An increase of the secretion was never observed as the result of the removal of the cortex.

*Frontal cross sections.* As appears from the table the gastric secretory response remains after cross sections localized rostrally to the hypothalamus. Attempts to study the effect of sections through hypothalamic regions failed as all dogs, where the sections included this region, died.

10 dogs were decerebrated. The height and the extent of the sections appear from fig. 1 and 2. In three of the dogs (no. 14, 23 and 43) a significant gastric secretion was induced by insulin. The secretory response was markedly reduced as compared with the secretion before the decerebration and the onset of the secretion delayed. In one test, dog no. 19 (very incompletely decerebrated) showed a significant secretory response.

### Discussion.

It is not known how hypoglycemia initiates vagal secretory impulses to the gastric mucosa. Theoretically it is conceivable that vagal secretory impulses are induced either by the removal of (cortical) inhibitory influences or by the activation of sub-cortical regions.

Our results show that the gastric secretory response to insulin remains in decorticate dogs. In most of our decerebrate animals the secretory response disappeared. In three dogs, however, a significant gastric secretion was observed.

The fact that hypoglycemia initiates a gastric secretion in a decerebrate animal indicates that hypoglycemia is able to activate vagal medullary structures. However, these observations do not justify the conclusion that in the intact animal the gastric secretory response to hypoglycemia is due primarily to the activation of these nervous structures. The small secretory responses and the delayed onset of the secretion in the decerebrate animals might indicate that in the intact animal the secretory impulses take their origin from nervous structures more sensitive to hypoglycemia. According to the claims of LA BARRE (1933) the gastric secretion induced by hypoglycemia is due to the activation of "thalamic regions". This conclusion is based on the observations that the gastric secretory response is abolished by barbiturates and disappears after ablation of the hemispheres and the thalamic regions of the dog. In our view it is not unreasonable to assume

that in the intact animal the secretory impulses emanate from the hypothalamic region. Electrical stimulation of this region is claimed to induce gastric secretion (HESLOP 1938). According to KRIEG (1932) in the rat efferent fibres run in the dorsal longitudinal fascicle from the hypothalamus to the dorsal motor nucleus of the vagus.

### Summary.

The gastric secretory response to hypoglycemia produced by insulin remains after decortication. After decerebration the secretory response disappears in most animals. In a few animals a reduced and delayed response remains.

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### Appendix.

The influence of hypoglycemia on gastric motility was observed in intact, decorticate and decerebrate gastric fistula dogs.

### Technique.

A rubber balloon was pushed into the stomach through the esophagus or through the gastric fistula. The balloon was filled with air to a pressure of 8—10 cm water, connected to a water manometer and the intragastric pressure continuously recorded on a smoked drum. In order to reduce the spontaneous gastric motility the dogs were fed in the morning. Two hours later the stomach was emptied through the fistula, washed and after another hour insulin was given



Fig. 3. Dog 7 kg. The influence of hypoglycemin on gastric muscular tone and motility.  
↑ Insulin I. V. 0.35 IU/kg.

intravenously. Blood sugar was determined every 15 minutes throughout the experiment.

Confirmatory to the reports of MULINOS (1933), NECHELES et al (1940) and others, in intact dogs hypoglycemia was observed to produce an initial inhibition followed by a sustained augmentation of gastric motility. One half to two minutes after the injection of a moderate dose of insulin. 0.2—1 unit per kg body weight, a sudden drop of gastric tonus and gastric peristalsis occurred. After 10—15 minutes the gastric tonus gradually rose above the initial level. Concomitantly the amplitude and frequency of the rhythmic movements of the stomach increased and remained markedly augmented for 3—4 hours (see fig. 3).

Decortication did not significantly change the motility pattern of the stomach. Spontaneous movements as well as the motor response to hypoglycemia remained unaltered. In three completely decerebrate dogs (45, 46 and 53 in fig. 1 and 2) spontaneous motility of the stomach was absent or markedly reduced. Hypoglycemia did not evoke increased gastric tonus or peristalsis in these dogs.

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# The Electrodialytical Method for Total Cation Determination.

## A Critical Study.

By

EVA WALAAS and OTTO WALAAS.

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The determination of cation with the aid of electrodialysis and the consequent amalgamation that follows is first described by GIBBS (1880) and carried out experimentally by SMITH (1903) and HILDEBRAND (1907). STODDARD (1927) developed a micro-method for the determination of sodium and potassium in biological material after ashing. The anode and the cathode cells were in this case separated by a glass wall which was finely perforated so that the cations passed through during the electrolysis and were amalgamated in the cathode cell, the content of which was thereafter titrated with sulphuric acid. ADAIR and KEYS (1934) allowed the cations to pass through a collodium membrane before they became amalgamated in the cathode cells. Direct analysis of the serum without the removal of the proteins became therefore possible, but each membrane had to be controlled for impermeability by the proteins before use.

KEYS (1936) modified the method by applying cellophane membranes, which are impermeable against proteins. NIELSEN (1940) also reports good results with cellophane membranes, and introduces a new type of apparatus. HOLM-JENSEN (1943) finds in contrary to this, a high and inconstant blank value with cellophane, and therefore prefers collodium which has a low blank

value. ØRSKOV and RATJEN (1947) have worked out a method applying electrolysis without dialysis through a membrane, the amalgames being transported to the cathode in a rocking-type apparatus.

From the literature it is evident that the electrodialysis method for the cation determination has given good results for sodium and potassium, a somewhat incomplete result with regard to calcium, and little or no yield with magnesium.

STODDARD reports the extraction of sodium and potassium to be 99 %, ADAIR and KEYS 99—100 %, NIELSEN 100 %, HOLM-JENSEN 98—100 %, BROCH (1945) 100 %, ØRSKOV finds 100 % for sodium, and 98—99 % for potassium. In the case of calcium KEYS reports a yield of 95 %, HOLM-JENSEN 100 %, whilst ØRSKOV's method gave 90 %. All the authors find little or inconstant yield from magnesium. HOLM-JENSEN finds 20—50 % independent of other ions in the solution. BROCH finds 5—15 %, ØRSKOV did not succeed in extracting magnesium with his method.

The accuracy of the method with regard to sodium and potassium assays is reported by STODDARD to be 2 %, KEYS 1 %, BROCH finds a mean error of  $\pm 0.68$  %. HOLM-JENSEN finds a maximum difference with the serum assays of 3 m.eq. or 2 % of the total bases by the appliance of collodium membranes, but a somewhat greater error with the cellophane membranes.

When analysing biological material the greatest error is caused by the magnesium content. This plays a comparatively small part in serum with a magnesium content of 2—3 m.eq. per litre, but is of great importance with the assays of ashed biological organs, where the magnesium content is considerably higher, 10—20. m.eq.

The accuracy of the method has otherwise varied somewhat with the applied types of membranes. A high and variable blank value of the dialysis membrane will influence the adaptability of the method to the micro assays. The types of membrane have therefore been continuously varied in the different modifications.

If the method is to give a real expression for the total bases in a biological material applied as a micro method the following conditions must be demanded:

1. Complete extraction of sodium, potassium, calcium and magnesium.
2. A comparatively low and constant blank value with the applied types of membrane.

### 3. No extraction of bases from the proteins.

This paper contains a survey of these factors.

## Technique.

### *Total bases.*

The electrodialysis technique described by NIELSEN has been employed with some modifications.

An electrolysis apparatus of Holm-Jensen's type was used, but with anode and cathode tubes of the type used in Nielsen's apparatus.

For each electrolysis cell a 200 voltage was established between the poles from a rectifier. The apparatus was constructed for electrolysis for up to 10 tests simultaneously. The assays were carried out in 0.2 ml. serum or saline solutions.

The following measures were taken in order to achieve the greatest possible accuracy:

1. The cleansing of the mercury was carried out with  $\text{HNO}_3$ , tap-water and distilled water, as described by Van Slyke.

2. The anode beakers and the cathode tubes were cleansed with chromisulphuric acid, and afterwards very thoroughly washed. Finally they were rinsed several times with glass distilled boiled water.

3. 10 ml. of the glass distilled water which prior to this had been boiled in order to remove the ammonia (cpr. NIELSEN) was placed in the anode beakers. At one time the glass distilled water was redistilled in order to remove small traces of cations, but this did not reduce the blank value any further. The blank value increased considerably if the water was not boiled.

4. The cellophane membranes were softened 5—10 minutes in glass distilled boiled water and were fixed with washed thread and with collodium along the edge. It was tested for leakage before use.

5. Repeated washing of the hands during preparation of the membranes and avoidance of contact with the membranes and parts of glass which were submerged in the analysis.

6. When the electrolysis had finished the current was broken by the cathode tubes being lifted up from the analyses. Thereby polarisation was avoided which might have lowered the extraction result.

Furthermore,  $n/100 \text{ H}_2\text{SO}_4$  was used in the cathode tube, and titration was carried out with  $n/100 \text{ NaOH}$  with methyl red—methylene blue as indicator.

According to our experiences the method requires a very meticulously carried out technique in order to give good results.

## Assays of Saline Solutions.

### *The accuracy of the Method.*

The accuracy of the method was registered by electrodialysis of sodium and potassium solutions (Table I) with the average results 99.4 % and 100.3 % respectively and with a standard deviation of  $\pm 1.4$  % and  $\pm 1.6$  % respectively. The electrodia-

lysis process itself thus gives complete extraction as far as these ions are concerned. The individual dispersion in the analysis then partly depends on the factors which are caused by the electrodiagnosis process, and which also manifest themselves in the blank value, and partly on errors in the pipetting and titration.

**Table I.**  
*Total Base Determinations.*

	No. of assays	Mean value m.eq./l.	Standard deviation m.eq.	Standard error of mean %
<i>Blank value</i>				
Cellophane membrane .....	48	1.4	$\pm 0.22$	$\pm 0.03$
» (Corrected for analysis).....			$\pm 1.1$	$\pm 0.2$
Acetate cellulose membrane ..		1.4		
Parchment membrane .....		6.6		
Collodium membrane .....		0.2		
NaCl, 100 m.eq./l. ....	22	99.4	$\pm 1.4$	$\pm 0.3$
KCl, 100 m.eq./l. ....	16	100.3	$\pm 1.6$	$\pm 0.4$
Na-K-Ca-Mg, total. 153.8 m.eq./l. ....	21	153.5	$\pm 1.8$	$\pm 0.3$
Serum I .....	6	158.4	$\pm 1.2$	$\pm 0.3$
Serum I .....	8	157.8	$\pm 1.0$	$\pm 0.3$
Serum II .....	7	162.0	$\pm 1.0$	$\pm 0.3$
Serum III .....	7	158.2	$\pm 1.3$	$\pm 0.4$
Serum III, ashed .....	7	157.2	$\pm 1.4$	$\pm 0.5$

### Blank Value.

The blank value is caused by the following factors:

1. Cations extracted from the glass wall in the anode beaker.
2. Cations in the distilled water.
3. Cations as contamination in the mercury in the anode beaker.
4. Cations from the membrane.

With the application of the same type of cellophane membranes, determination of the blank value in 48 assays was carried out with an average reading of 1.4 m.eq., and standard deviation of  $\pm 0.22$  m.eq. When the latter is corrected for the analysis of 0.2 ml. this will correspond to a standard deviation of  $\pm 1.1$  m.eq. Thus this becomes only a little less than the standard deviation of the analysis of saline solutions. Consequently the dispersion of the values of saline solutions is only for a smaller part caused by errors in pipetting and titration, while the greatest error is to be found in the electrodiagnosis process itself.



Furthermore, the application of various types of membranes during the electrodialysis is investigated. As is evident from Table I, the blank value varies considerably with the type of membrane applied, from the maximum 6.6 m.eq. with parchment, down to 0.2 m.eq. with collodium. The greatest part of the blank value is thus caused by cations from the membrane itself.

The parchment shows the largest blank value and as the membranes also are stiff and difficult to prepare, these membranes are little suited.

Various acetate cellulose membranes were tried — ready-made membranes as well as membranes which we prepared ourselves from acetate cellulose dissolved in acetone, which was later evaporated. They showed a rather constant and low blank value but were frequently torn during electrolysis of saline solutions and were, therefore, not suitable. Collodium has the advantage that the blank value is very small but on the other hand it takes a long time to prepare these membranes and they are easily torn with a consequential unsuccessful analysis. Cellophane membranes are, therefore, found to be the most suitable from the types tried. The fabricated membranes are not homogeneous and cause the variation in the blank value observed. Each new type of cellophane which is taken in use should, therefore, be tested first. The type which we have found most suitable is the cellophane "Alofan"<sup>1</sup>, with a thickness of 0.02 mm.

The other factors in the blank value, the distilled boiled water and the mercury were investigated by varying the amounts, but this was found not to have any influence on the blank value. The cations from the glass wall were investigated by comparing new anode beakers with old ones before they were taken in use but no difference was detected here either.

It must, therefore, be presumed that the factor which is responsible for the major variation in the analysis is the membranes. The cellophane which was used in our experiments gives satisfactory accuracy in micro methods.

### Time of Electrolysis.

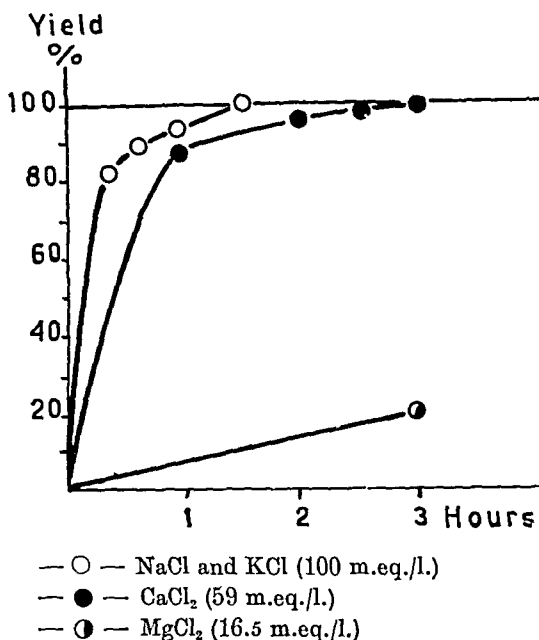
KEYS as well as NIELSEN state the necessary time of electrolysis to be 1 hour, but claim that most of it is transferred after 30

<sup>1</sup> Manufactured by A/B Nordisk Silkecellulosa, Norrköping.

minutes. HOLM-JENSEN states 1 hour for alkaline metals but 3 hours electrolysis to transfer calcium completely. The time of electrolysis in our experiments is tabulated in Diagram I. After 1 hour 90 % of the sodium and potassium is extracted, and 100 % extraction is reached after 1  $\frac{1}{2}$  hours. 1 hour electrolysis which is used by most of the workers is, therefore, somewhat insufficient. Furthermore, we are able to verify the experience of HOLM-

Diagram I.

*Cation Extraction in Relation to the Time of Electrolysis.*



JENSEN that the calcium is more slowly transferred — after 1 hour 85 %, after 2 hours 95 % and then after 3 hours 99 % to 100 %. When KEYS reports 95 % extraction for calcium, the reason is most likely too short a time of electrolysis.

### Determinations of Magnesium.

The determinations of magnesium in our preliminary experiments gave little and variable results, and thus verified the experiences of earlier workers. By the analysis of a MgCl<sub>2</sub>-solution of 16.5 m.eq./l. an average yield of 20 % was found with variations from 3 % to 58 % in the various analysis, with a time of electro-

lysis of 3 hours. The reason for this low yield in magnesium chloride solutions may depend on 2 factors:

1. The lack of amalgamation of magnesium.
2. Slow movement of the magnesium ions through the solution and the membrane.

With regard to the first factor, it is known that magnesium is slowly and incompletely amalgamated. It is, therefore, according to HOLM-JENSEN most likely to presume that  $\text{Mg}^{++}$ , after penetrating through the membrane, is transferred to Mg-Hydroxyd by the corresponding capillary layer of water between the membrane and the mercury. The precipitated  $\text{Mg}(\text{OH})_2$  will then be found as a film on the membrane, and by the transferring of the content of the cathode tubes to the titrating flask, only part of the precipitated hydroxide is removed. Thus the low and inconstant values are brought about. By the electrolysis method of ØRSKOV, no yield is obtained from the magnesium solutions, and this verifies the given explanation. We then had the possibility of including the magnesium ions during the titrations by cutting the membrane, and transferring this to the titration flask together with the contents of the cathode tube. During shaking, the following reaction will then take place:  $\text{Mg}(\text{OH})_2 + \text{H}_2\text{SO}_4 = \text{Mg SO}_4 + 2 \text{H}_2\text{O}$ . The membrane was firstly thoroughly rinsed on the outside by submerging it twice in distilled water, then it was cut at the lower edge of the cathode tube and transferred into the titration flask which was thoroughly shaken, after which the titration was carried out in the usual manner. As is evident from Diagram II we then obtained a yield of 85 % against 20 % with the usual technique. The incomplete amalgamation is, therefore, the major reason for the unsatisfactory yield of  $\text{Mg}^{++}$ .

As one did not obtain quite the same yield as for the other cations, it is possible that also the second factor — slow moving speed — played a part.

It is known that by the electrolysis of the aqueous solutions of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , the mobility of all these ions is found to be approximately the same. The mobility of the  $\text{Mg}^{++}$  ions through the aqueous solution in the anode beaker is thus equally as rapid as for the other cations.

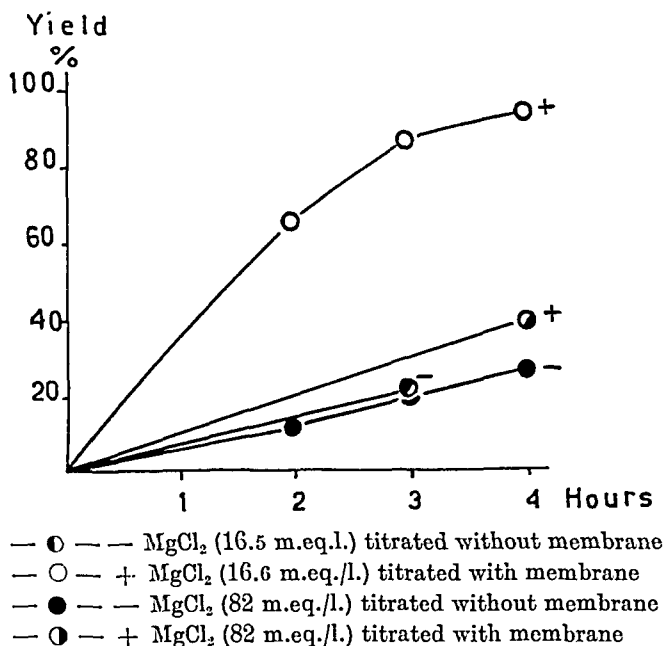
The mobility of the cations through membranes depends on a number of physical chemical processes such as the size of the pores, the solubility of the ions in the membrane, adsorption phenomena such as shown by WEISER (1930), as well as the

electrical potentials of the membranes affecting the transportation of the ions reported by LØDDESEØL (1932) and others.

The penetration speed through the cellophane membranes is demonstrated by experiments in Pauli's three-cell electro dialysis

Diagram II.

*Electrodialysis of  $MgCl_2$  in the Total Base Apparatus.*



apparatus, where a comparison between the speed of movement of  $Na^+$  and  $Mg^{++}$  has been undertaken.

In these experiments with pure solutions of  $Na^+$  and  $Mg^{++}$ , the following methods have been applied.

#### *Sodium analysis.*

Sodium analyses were carried out by a polarographic method suggested by MAJER (1933), this method presumes the application of tetramethyl-ammonium hydroxyd as the supporting electrolyte but as this was impossible to obtain, choline was used instead, which showed a reduction potential by a voltage of about 2.5 volts. Open polarographic determination was used in a "Radiometer" polarograph. The calibration curve for sodium solutions showed a straight line for concentrations  $2 \cdot 10^{-2}$  to  $10^{-3}$  mol./liter.

Polarography took place at alkaline pH. The polarographic wave was found unaffected by changes in the pH between 7 and 12.

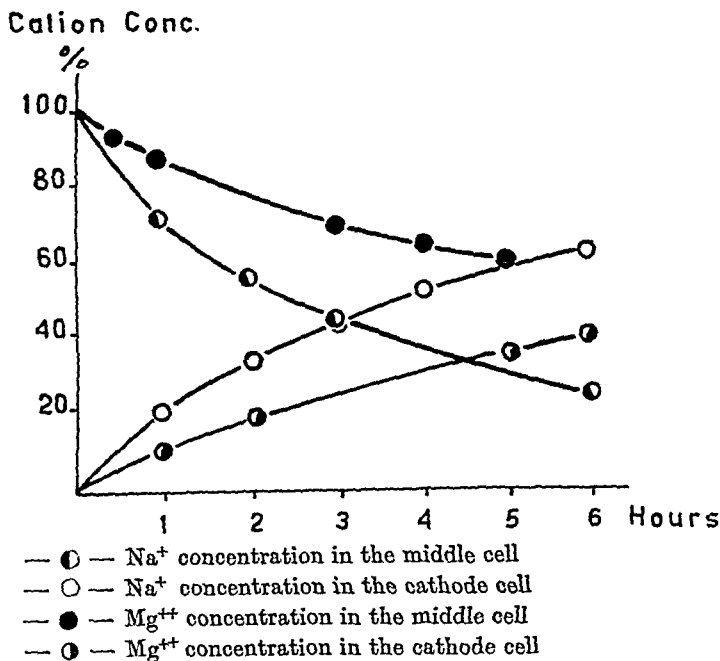
### *Magnesium assays.*

Magnesium assays were likewise carried out by a polarographic method suggested by STONE and HOWELL FURMAN (1944). The principle is a polarographic determination of 8-hydroxyquinoline before and after the precipitation of the magnesium salt. The fall in the height of the wave which takes place is a measurement for the amount of magnesium which is precipitated in the buffer solution by pH 10. The method will be described in detail in a further publication, where it is worked out for the determination of magnesium in biological material.

In both methods the general precautions are taken for polarography according to KOLTHOFF and LINGANE (1941). By analysis of a series of known solutions the accuracy in regard to sodium and magnesium analysis was found to be  $\pm 2\%$ .

### Diagram III.

#### *Electrodialysis in Pauli's Apparatus.*



Electrodialysis experiments of  $n/10$  solutions of cations in the middle cell against distilled water or against  $n/10$  HCl in the cathode cell have been carried out (Diagram III).

Mg<sup>++</sup> moves slower than Na<sup>+</sup> through the cellophane membranes. It will be further seen that both ions are delayed during the trans-

port through the membrane, the fall of the concentration in the middle cell taking place at a greater speed than the increase of the concentration in the cathode cell. The cations are accordingly delayed during the electrolysis through the membrane so that retardation takes place of the movement to the cation cell. After five hours electrodialysis the fall of the concentration in the middle cell is for  $\text{Na}^+$  72 % and for  $\text{Mg}^{++}$  40 % while the concentration in the cation cell is for  $\text{Na}^+$  58 % and for  $\text{Mg}^{++}$  33 %. The speed of movement for  $\text{Na}^+$  is accordingly nearly double as great as for  $\text{Mg}^{++}$ .

The fact that this slower membrane transportation also plays a rôle for  $\text{Mg}^{++}$  by determination of total cations in the total base apparatus is evident from Diagram II. The electrolysis of a solution of 16.5 m.eq./l.  $\text{MgCl}_2$  shows increased yield when the time of electrolysis is prolonged: 2 hours 64 %, 3 hours 85 % and 4 hours 93 %. By analysis of a stronger solution: 82 m.eq./l.  $\text{Mg}^{++}$ , this factor plays an important part, as only 40 % yield was found after 4 hours electrolysis. This, however, plays no practical rôle in biological work as one has no higher concentration of  $\text{Mg}^{++}$  than 10–20 m.eq./l. in biological material.

By electrolysis of 16.5 m.eq./l.  $\text{Mg}^{++}$  solution, one does not reach 100 % yield even if the electrolysis time is prolonged over 4 hours. The reason may be that  $\text{Mg}^{++}$  during the electrolysis also moves to the anode. LØNDESØL (1932) has shown that  $\text{Mg}^{++}$  during electrolysis can partly appear as a complex ion and move to the anode.

The conclusion of our experiments with  $\text{Mg}^{++}$  shows that the incomplete amalgamation as well as the slow movement speed through the membranes are of importance for the unsatisfactory yield with the electrodialysis method. During the application of the method we have, therefore, introduced two modifications.

1. Increasing of the time of electrolysis to 3–4 hours.
2. Cutting off of the membrane and transferring the same to the titration flask after completed electrolysis.

When the method is applied in this way, one can figure on the yield of 100 % for  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$  and 90 %–95 % for  $\text{Mg}^{++}$ .

Table I shows the electrolysis of a combined saline solution corresponding to serum:  $\text{Na}^+$  142 m.eq.,  $\text{K}^+$  5 m.eq.,  $\text{Ca}^{++}$  5 m.eq.,  $\text{Mg}^{++}$  1.8 m.eq., total 153.8 m.eq./l. 21 assays showed the mean value of 153.5 m.eq., in other words complete agreement with that which was expected.

### Serum Analyses.

The importance of titration with and without membranes transferred to the titration container, was controlled in serum analyses (Table II). The values were in 6 out of 7 cases as an average 1.6 m.eq. higher when titrated with the transferred membrane. If it is calculated with a Mg content in the serum of 2

Table II.

*Total Base Determination of the Sera.*

	Titrated without mem- brane	Titrated with transferred membrane	Balance
Serum 1 .....	161.8 m.eq./l.	162.4 m.eq./l.	+ 0.6 m.eq.
Serum 2 .....	157.8 »	158.4 »	+ 0.6 m.eq.
Serum 3 .....	155.6 »	157.7 »	+ 2.1 m.eq.
Serum 4 .....	157.8 »	161.5 »	+ 3.7 m.eq.
Serum 5 .....	154.8 »	158.4 »	+ 3.6 m.eq.
Serum 6 .....	153.4 »	155.4 »	+ 2.0 m.eq.
Serum 7 .....	154.0 »	153.1 »	- 0.9 m.eq.
Mean	156.5 m.eq./l.	158.1 m.eq./l.	+ 1.6 m.eq.

m.eq. this is in good agreement with the calculated yield of 20 % by the usual method and 90 % with our modification.

The accuracy of the method was investigated by a series of analyses in the same sera (Table I). The standard deviation for these analysis is  $\pm 1.0$  —  $\pm 1.3$  m.eq., and dispersion is even less than for the saline solutions. HOLM-JENSEN states the difference of 3 m.eq. between duplicate analyses with his method using collodium membranes, and ØRSKOV reports a standard deviation for serum of  $\pm 1.6$  m.eq. in his method. The electro dialysis with cellopane membranes has, in othher words, given equally as accurate results in our experiments.

In the analysis of serum one has the possibility that the proteins influence the results, partly by the proteins binding fixed bases in complexes so that they do not act as cations and partly by the possibility that the proteins split basic groups especially guanidine which acts as a cation. In the first case one will obtain too low, in the latter case too high, values by determination of total fixed bases. Analyses of sera with and without ashing illustrate this

condition (Table III). As an average one finds slightly higher figures without ashing but the difference is inconstant and does not exceed the limit of the errors of the method.

Table III.  
*Serum Analyses.*

	Direct deter- mination	After ashing	Balance m.eq.
Serum 1 .....	158.8 m.eq./l.	156.4 m.eq./l.	— 2.4
Serum 2 .....	161.2 "	157.2 "	— 4.0
Serum 3 .....	160.4 "	159.2 "	— 1.2
Serum 4 .....	158.8 "	160.4 "	+ 1.6
Serum 5 .....	158.0 "	160.4 "	+ 2.4
Serum 6 .....	163.6 "	162.8 "	— 0.8
Serum 7 .....	158.5 "	157.2 "	— 1.3
Mean	159.9 m.eq./l.	159.1 m.eq./l.	— 0.8

### Summary.

1. The electroalytic method for the determination of total cations gives 100 % yield for  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ .
2. For  $\text{Mg}^{++}$  a modification is reported consisting in transferring the membrane to the titration flask after completed electrolysis. Hereby the yield is increased from 20 % to 90 %—95 %.
3. The electrolysis time for  $\text{Na}^+$  and  $\text{K}^+$  must be  $1\frac{1}{2}$  hours, and 3—4 hours for  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ .
4. By the application of cellophane membranes with analysis of sera, a standard deviation of  $\pm 1.0$  to  $\pm 1.3$  m.eq. was found and a standard error of mean of  $\pm 0.3$  %.
5. The proteins did not influence the yield of cations in the serum analyses.

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## Physiological Variations and Normal Values of the Total Bases in Blood Serum.

By

EVA WALAAS and OTTO WALAAS.

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In 1931 PETERS and VAN SLYKE reached the following conclusion as a result of investigations of the total bases in serum: "One of the most jealously guarded constants of the organism is the total electrolyte concentration of the blood plasma." This view later became generally recognized. According to PETERS and VAN SLYKE the individual anions may alter in a series of physiological and pathological conditions: "But the entire course of distribution in anion relationships may be run with little or no change in the concentration of the total base." Alteration of the total bases occurs only in severe pathological disturbances.

In this paper the investigations of the constancy of the total bases in connection with the physiological conditions are taken up to discussion. The problem is thus presented: Can variations in the total bases be found because of physiological changes in the metabolism, in particular such changes where individual variations on the part of the anions occur. The following conditions have been examined: 1. The effect of the position of the body. 2. Muscular work. 3. Fluid intake. 4. Hyperventilation. 5. Food consumption.

Investigations with regard to the total bases under these conditions are few, and it seems that the technique often was en-

cumbered by errors. In SENDROY's conclusion over electrolyte assays in serum (1938) it is stated that the technique in many earlier investigations is of doubtful accuracy, and that all analyses of the acid-base equilibrium in the blood therefore need renewed investigation and revision.

As a result of our experiments the normal values of the total base in relation to varied experimental conditions have been further stated.

### *Technique.*

#### *Total Cation Determination.*

An electrodialytic method worked out and published by the authors in this journal was used. (WALAAS and WALAAS 1949.)

#### *Chloride.*

The electrometric method for 0.2 ml. serum as suggested by LEHMANN (1944) was used. A modification has been introduced, the proteins being precipitated before the titration. In standard solutions the accuracy of the method was found to be  $\pm 0.2$  m.eq.

#### *Bicarbonate.*

Van Slyke's manometrical method was used.

#### *pH Determination.*

Electrometric determination with a glass electrode and a "Radiometer" apparatus was applied. Accuracy 0.02 pH units.

#### *Collecting of Samples.*

All samples were taken by venous puncture in the cubital vein after previously short-lasting stasis not exceeding 15 sec. Most of the samples were collected anaerobically with the aid of paraffin. liquid. The samples were then immediately centrifuged and the serum pipetted out and analysed the same day. In some cases where only the total cations were determined the samples were taken aerobically.

## **Body Position.**

Only a few investigations concerning the changes in the electrolytes by varying the body position are available. LILJESTRAND and WOLLIN (1913) and MAIN (1937) describe changes in the acid base balance by changing from a horisontal to an upright position: Decreased alveolar  $\text{CO}_2$  tension and slight increase of pH. These changes are presumed to be caused by hyperventilation and consequent alkalemia in the upright position. The changes are, however, temporary and MAIN states that already after 10 minutes in an upright position the same value as for the horisontal position is reached.

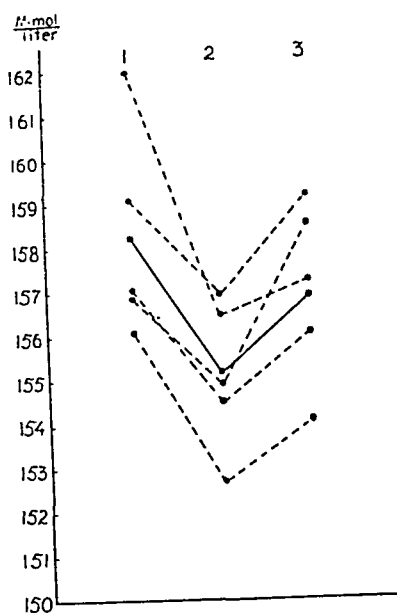


Diagram 1.

Total bases in the static experiment.

1. Standing. 2. Lying. 3. Standing.

— Average curve.

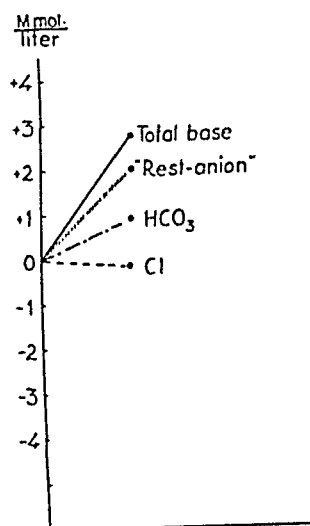


Diagram 2.

Electrolyte changes by varying the body position from lying to standing.

### Experimental.

In 5 experiments the total bases are determined at the time of changing from the upright to the horizontal position and again from the horizontal to the upright position. Sample no. 1 is taken during ordinary laboratory work, standing, walking. Sample no. 2 is taken after 30 min.'s horizontal position with complete relaxation. Sample no. 3 is again taken after 30 min.'s walking or standing position. (Diagram 1.) In all the experiments there is a decrease from the standing to the horizontal position averaging from 158 m.eq. to 155 m.eq., followed by an increase to an average of 157 m.eq. in the upright position.

In 10 experiments the changes in the electrolytes were studied more closely when moving from the horizontal to the upright position (Diagram 2). All experiments showed an increase in the total bases averaging 2.6 m.eq.

Chloride and bicarbonate on the other hand show small and varying changes. Bicarbonate shows in most of the cases a slight increase when changing from the horizontal to the upright position, and in 2 experiments a somewhat larger increase was found of 2.5 and 3.3 m.eq. Chloride shows in most of the experiments

a slight decrease. Bicarbonate and chloride vary in the individual experiments in inverse directions such as could be expected according to the influence of the Donnan effect on the distribution of the diffusable ions in the blood.

### Discussion.

The body position effects the total bases in serum in a constant direction: The total bases are decreased when changing from upright to horizontal position and they increase again when changing to an upright position.

On the other hand the changes in the examined anions are uncharacteristic and offer no explanation for the variations of the total bases observed. The bicarbonate shows in most of the cases in the upright position a slight increase, but whether this is accompanied by alkalemia cannot be decided, as the pH has not been measured. The changes in the chlorides are small and move in the opposite direction from the bicarbonate so that the total of these two is kept almost unchanged.

One has furthermore, in these investigations, carried on the calculation of the group "Rest anions" which accordingly include phosphates, sulphates, organic acid anions and the protein anions. This group shows an increase in the upright position in all the experiments and therefore evidently gives the explanation for the increase in the total bases. A primary increase of the latter may be considered unlikely. Essential changes in the phosphates, sulphates and organic acid anions are not very probable in these statical experiments.

The influence that the body position has on the serum proteins is well known however. In the upright position the protein concentration will increase from 10—20 % in proportion to the horizontal position, as is shown by THOMPSON, THOMPSON and BAILEY (1928), LANGE (1946) and others. At the same time a corresponding decrease in the plasma volume is found. The major cause for this is believed to be the increased hydrostatic pressure in the upright position together with increased filtration pressure in the capillaries. Parallel with the increase in the protein concentration, an increase in the proteins base-binding capacity takes place.

The calculation of the base-binding capacity of the serum proteins was in this paper carried out on the basis of formula

given by VAN SLYKE, HASTINGS, HILLER and SENDROY (1928)  $BP_s : 0.104 \times \text{Protein} \times (\text{pH} - 5.08)$ . This is stated to be normally at an average of 17 m.eq./l. From the formula it will be seen that the base-binding capacity increases with the protein concentration. An increase of 10—20 % as in our experiments accordingly means an increase of the base-binding capacity of 2—3 m.eq. This value is in good agreement with the increase observed of the "Rest anion" group with an average of 2.1 m.eq.

BROCH (1945) has stated that VAN SLYKE and coworkers' formula for the base-binding capacity is not valid in some pathological conditions, possibly because of qualitative changes in the composition of the proteins. There is no certain evidence on the other hand that the formula is not valid in physiological conditions.

It seems therefore feasible to presume that the increase in the total bases in the upright position is primarily caused by an increase in the concentration of the serum proteins.

### Muscular Work.

The well-known electrolyte changes in serum by muscular work consist of an increase of the lactic acid and a decrease in the bicarbonate. The  $\text{CO}_2$  being ventilated by respiration as an expression of one of the organism's fundamental buffer mechanisms. The observations of the relative changes in lactate and bicarbonate have been partly contradicted. BARR, HIMWICH and GREEN (1923) find in the venous blood a sometimes small, and a sometimes larger decrease in the bicarbonate as compared with the increase in the lactate. JERVELL (1928) finds a larger decrease in bicarbonate with heavier work, and DILL, TALBOTT and EDWARDS (1930) likewise find a larger decrease in bicarbonate than the increase of lactate by light work. In arterial blood BOJE (1935) finds that the decrease in bicarbonate is equivalent to the increase in ether soluble acids. Another view is taken by TURRELL and ROBINSON (1942) who find in arterial blood a decrease of the bicarbonate and an increase in lactate equivalent up to 4 m.eq./liter, but above this limit the lactate increases more than the decrease of the bicarbonate. They presume that the surplus of the lactic acid is neutralized by bases discharged from proteins. The maximum lactic-acid content was found to be 22 m.eq.

The pH changes in serum by light muscular work are, according to investigations by CULLEN and EARLE (1929), very small.

With heavier work acidosis occurs. TURRELL and ROBINSON have found as the lowest value pH 6.97.

During muscular work there furthermore occurs a decrease of the water content of the serum and as a result of this a relative increase in the protein concentration. According to investigations by DILL, TALBOTT and EDWARDS (1930), COVIÁN and KROGH (1934), BLEGEN (1940), LANGE (1946) the increase of the protein concentration varies from 5—20 % increasing with the degree of muscular work.

Much more uncertain is the information about changes in the other electrolytes in serum during muscular work. PETERS, BULGER, EISENMAN and LEE (1926) state in a single experiment an increase of the total bases, but the increase of 14 m.eq. that was found seems remarkably large. SCHULTZ, HASTINGS and MORSE (1935) report decrease in the total bases, but MORSE and SCHULTZ (1940) find an increase and the technique is stated to be somewhat uncertain. DILL, TALBOTT and EDWARDS find increase of the total of sodium, potassium and calcium by 2—3 m.eq. during work. COVIÁN and KROGH as well as BLEGEN find increase in the total osmotic pressure and chlorides. Increase of chlorides is also found by DILL, TALBOTT and EDWARDS, and MORSE and SCHULTZ, but PETERS, BULGER, EISENMAN and LEE find unchanged chlorides. Increase of phosphates is reported by HAVARD and REAY (1926), JERVELL, and DILL, TALBOTT and EDWARDS.

The somewhat variable results may, apart from the technique of the analysis, depend on the variations in the nature and size of the muscular work.

### Experimental.

The effect of the muscular work on the serum electrolytes is studied by ergometer bicycle experiments. An ergometer bicycle as described by JERVELL (1929) has been used. The period of work was 10 min., and the experiments included a group with light work, 500 kgm per min., and one with heavier work, 1,000 kgm per min. 3 persons were used in the experiments in each group. In the first group they were all quite unstrained at the end of the work, but distinctly exerted in the second group at the end of the heavier work. The blood samples were taken while the work was in progress. The results are given in Diagram 3.

In the group "light work" changes were small. There was no distinct decrease of bicarbonate, whereas a reduction of the pH

was found in 2 experiments to pH 7.25. In these experiments the total bases show a small increase but a slight decrease in the third experiment. Apart from a slight acidosis in 2 experiments the changes in the electrolytes are thus insignificant and uncharacteristic. The muscular work has thus been too slight to produce any increase in the lactic acid in the blood.

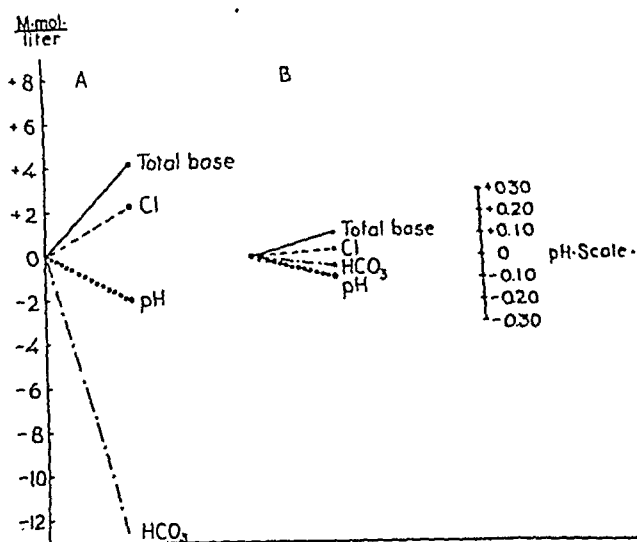


Diagram 3.

Electrolyte changes during muscular work. Average values.

A: 1,000 kgm/min. in 10 minutes.

B: 500 kgm/min. in 10 minutes.

After the heavier muscular work the characteristic changes are found with a decrease of bicarbonate at a maximum of 17.5 m.eq./liter, and occurrence of acidosis with pH reduced to 7.12.

Otherwise an increase of total bases averaging 4.5 m.eq. is found in all experiments as well as an increase of chlorides averaging 2.5 m.eq. An increase of these electrolytes must therefore be regarded as characteristic with heavier muscular work. When earlier investigators have found variable results this most likely depends on technical errors in the analysis.

### Discussion.

The most interesting finding in the experimental group "heavy work" is the increase in the total bases which occurs in all the experiments. In order to find the explanation for this the changes of the other electrolytes have been studied. In the anion group



a considerable increase in the lactic acid has taken place with the occurrence of acidosis, and a consequent reduction of the bicarbonate buffer in the blood.

Increase of the group "Rest anion" may be accounted for by several factors:

1. The increase of the lactic acid is quantitatively the most important factor.

2. Inorganic phosphate increases during muscular work. DILL, TALBOTT and EDWARDS state increases of 0.4 m.eq.

3. The base-binding capacity of the proteins changes during muscular work. During work an increase of the protein concentration at an average of 18 % is found in our experiments and simultaneously an average decrease of pH of 0.20. These two factors will then influence the  $BP_s$  in the opposite direction. Calculation after VAN SLYKE's formula in the two cases shows an average increase of the  $BP_s$  of 1.3 m.eq. in our experiments.

With light muscular activity no reduction in the bicarbonate was found, but on the other hand a decrease in the pH in two cases was discovered. This is contradictory to investigations by EARLE and CULLEN (1929) in arterial blood, where a slight increase in the lactic acid was found after light muscular activity which was easily neutralized without considerable pH reduction. Our findings indicate that the conditions in venous blood vary. HASTINGS, DILL and EDWARDS (1936) found increase of the  $CO_2$  content in the tissue during work and such an increase may explain the decrease of pH in venous blood.

The increase in the chlorides after heavy muscular work can probably be explained by the Donnan theory, with the transfer of chlorides from the red corpuscles to the serum.

The increase in total bases after muscular work must be regarded as a compensation in order to neutralize the increase which has taken place of the total anion content. The increase in chloride is hereby compensated as well as the part of the group "Rest anion" which is not bound by the bicarbonate buffer. Here it is a question of increase in the phosphates, proteinates and the part of the lactic acid and other organic anions that are not neutralized by Na-bicarbonate. The increase in the total bases that was found during muscular activity in our experiments, an average of 3 %, is relatively larger than the simultaneously occurring reduction of the water content, which was found to be at an average of 1.3 %. Bases must therefore have been introduced from the buf-

fers of the tissue fluid in order to maintain the electrolyte neutrality in the blood during heavy muscular work.

### Fluid Intake.

The typical change to be expected after drinking water is a decrease in the serum electrolytes, showed among others by PRIESTLEY (1921), FINDLAY and WHITE (1937) and BLEGEN (1940). The cause for this decrease of the electrolytes is presumed to be an excretion of NaCl in the intestine so that the water may be absorbed as a saline solution. BLEGEN found after drinking water a decrease of the total osmotic pressure as well as the total bases and chlorides. The decrease of both the latter were somewhat variable however, and not always corresponding to the reduction in osmotic pressure.

### Experimental.

The effect of the intake of water is examined in fasting persons after drinking 1 liter of water. The arrangement of the experiment was as follows:

1. Blood sample was taken from a fasting person before the experiment. Immediately afterwards he drank 1 liter of temperate water in the course of 5 min.

2. Blood sample was taken 30 mins. afterwards, and

3. Blood sample was taken 1 hour after the drinking of the water. (Diagram 4.)

In all 4 experiments a decrease was found in the total bases, averaging 2.8 m.eq. after 30 mins., and 3.6 m.eq. after 1 hour. Chloride showed, at the same time, a decrease of 2 and 2.5 m.eq. in the two groups. Bicarbonate showed no, or in any case a quite insignificant, increase.

### Discussion.

Our findings are well in agreement with earlier investigations of the serum electrolytes after drinking of

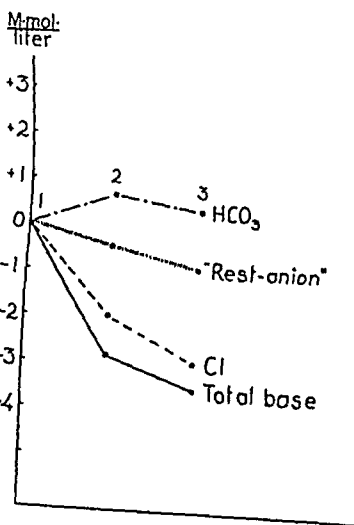


Diagram 4.  
Electrolyte changes after drinking of water.

1) Fasting. 2)  $\frac{1}{2}$  hour after 1 liter of water. 3) 1 hour after 1 liter of water.

water, with the conclusion that a decrease of the NaCl in serum is produced. In our experiments the decrease of the total bases is a little larger than the decrease of the chlorides. The group "Rest anion" shows therefore a slight decrease in all experiments. It is possible that this is caused by a decrease of either protein or electrolyte anions other than chlorides. PRIESTLEY (1921) as well as BALDES and SMIRK (1934), and BLEGEN (1940) found no uniform change in the serum proteins after drinking of water. In some cases there is an increase and in some cases a decrease in the concentration.

The decrease in the "Rest anion" group cannot therefore be placed in relation to changes of the proteins. Excretion of other salts in the intestine is more likely, such as phosphate and sulphate anions bound to base.

Statistical calculations of the values of total bases in the various groups shows that the difference between the mean values in Gr. I and II is  $2.8 \sigma$ . The difference between the mean values in Gr. I and III is  $3 \sigma$ . The decrease of total bases 30 mins. after drinking of water, is accordingly highly probable, and after 1 hour significant.

It should therefore be emphasized that the decrease of the electrolytes which has taken place both in the cation and the anion groups particularly by reduction of NaCl has not been followed by compensation in order to maintain the normal electrolyte level. Thus no increase is found in the bicarbonate or in any case only an insignificant increase.

### Hyperventilation.

The characteristic changes in the serum after short-lasting, voluntary hyperventilation are described among other by PETERS, BULGER, EISENMAN and LEE (1926), SHOCK and HASTINGS (1935—36), NIMS, GIBBS and LENNOX (1942) and RAPAPORT, STEVENS, ENGEL, FERRIS and LOGAN (1946), and manifest themselves by a decrease in the  $\text{CO}_2$  pressure and the  $\text{CO}_2$  content with the occurrence of alkalemia. These changes then cause tetany. The maximum decrease in the bicarbonate content is stated by SHOCK and HASTINGS and RAPAPORT and co-workers to be from 4—7 m.mol./l., while the maximum increase of pH is up to 7.71 in arterial blood. In venous blood the increase is somewhat smaller as reported by PETERS and co-workers, and NIMS and co-workers.

The other electrolyte changes in serum are less known. PETERS, BULGER, EISENMAN and LEE found no changes in the total bases, an increase in the organic acids and a rather unexpected decrease in chlorides. RAPAPORT, STEVENS, ENGEL, FERRIS and LOGAN have, in a very thorough investigation, recorded the electrolyte changes in arterial blood. Sodium was found increased by 3 m.eq./liter, potassium slightly reduced by 0.5 m.eq./l., calcium was unchanged. In the anion group an increase of chloride by 3 m.eq./l. was found, but organic phosphate is slightly decreased. NIMS, GIBBS and LENNOX found increase in the lactic acid both in the arterial and the venous blood. By long-lasting hyperventilation experiments, PETERS and co-workers found decreased bicarbonate content but normal pH, increased chloride values, while the total bases were normal.

### Experimental.

4 experimental persons have carried out voluntary hyperventilation during a short period of time, as a rule from 4—6 mins., until the occurrence of tetany symptoms. The hyperventilation has in particular consisted of an increase in the volume of tidal air, and not so much an increase of the frequency. In one of the persons no tetany occurred even after 15 mins. hyperventilation. The results are shown in diagram 5.

An alkalemia with an average increase of pH by 0.16 was found, and a maximum pH of up to 7.60. The bicarbonate was reduced on an average from 28.5 to 24 m.mol./l. Chloride showed an average increase of 1.5 m.eq. while the total bases were reduced at an average of 1.9 m.eq.

These findings are in good agreement with the changes found by RAPAPORT and co-workers in arterial blood where the total of sodium, potassium and calcium showed an average decrease of 2.8 m.eq./liter, while magnesium was not investigated. A decrease of the total bases in the serum of 1—2 % in arterial as well as venous blood may therefore be regarded as a typical change after hyperventilation.

Furthermore we have been able to investigate serum electrolytes in a person suffering from hysteria with a permanent hyperventilation. The patient was a 23-year-old girl who in 3 weeks had been suffering with a hyperventilation. In the night, during sleep, she respired normally but started to hyperventilate again as soon as she woke up. Slight signs of tetany in her arms could

be detected. 3 blood samples were collected from this patient, the first at 2.0 a.m. during sleep, the second at 3.0 a.m. after 1 hours hyperventilation. The third at 6 p.m. She had at that time hyperventilated throughout the whole day. Results are shown in diagram 5 B. During sleep subnormal values were found for bi-

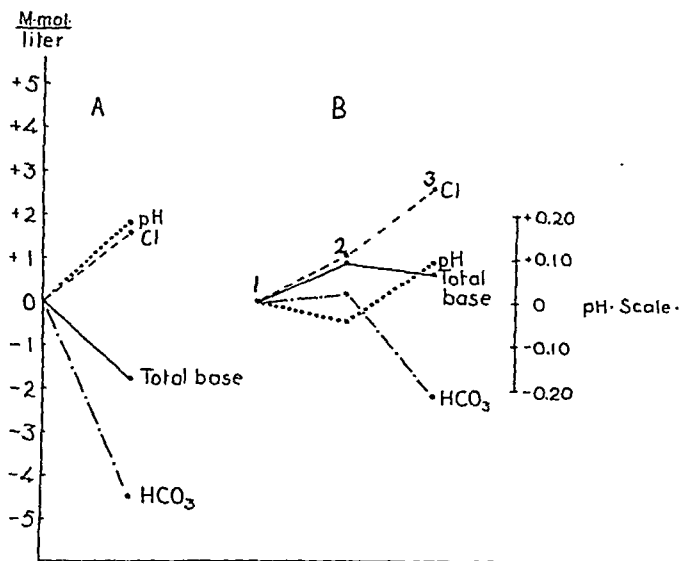


Diagram 5.

Electrolyte changes in hyperventilation experiments.

A. short-lasting.

B. Permanent.

1. During sleep.

2. 1 hours hyperventilation.

3. 12 hours hyperventilation.

carbonate: 21.6 m.mol., and above normal for chloride: 108.5 m.eq. But no alkalemia as pH was 7.38. One hours hyperventilation gave insignificant changes. After a whole day of hyperventilation on the other hand an alkalemia with pH 7.50 was found and bicarbonate now reduced to 19.4 m.mol. and chloride increased to 111.0 m.eq. In contrary to the conditions with short-lasting hyperventilation no certain changes in the total bases were found here, which had the constant value of 151 m.eq. It appears therefore as if the organism reacts differently in short-lasting and permanent experiments. The reduction which has taken place in the bicarbonate will, by short-lasting hyperventilation, be partly compensated by reduction in the total bases, while in permanent hyperventilation complete compensation in the anion group takes place, the chlorides being increased to values above the normal.

### Discussion.

The changes in the  $\text{CO}_2$  content which are found in short-lasting hyperventilation correspond to those described by NIMS, GIBBS and LENNOX in venous blood. The maximum pH increase which has been recorded is 0.23 and the maximum reduction in the bicarbonate is 6.3 m.mol.

The other electrolyte changes are of the same nature as described by RAPAPORT and co-workers in arterial blood. RAPAPORT has carried out the most complete study of the electrolyte changes after hyperventilation that exist, having investigated both the blood corpuscles and the serum. Complete agreement has been found between the experimental values and the calculations after the Donnan's Law, with an average reduction of the ion concentration in the serum of 1.7 %.

In our material no analysis of the blood-cell content of electrolytes has been carried out. But all our findings point in the same direction as RAPAPORT, and we find a reduction in the ion concentration of 1.2 %. This is thus somewhat lower than reported for arterial blood, but may be explained as stated by NIMS, GIBBS and LENNOX: The electrolyte changes in venous blood are less pronounced and are brought about more slowly than in the arterial blood.

The primary effect with the state of hyperventilation, namely reduction of bicarbonate, is thus compensated by two factors:

1. Increase of the other anions, particularly by transportation of chlorides from the red blood cells to the serum, furthermore a smaller increase of the lactic acid and the protein anions is likely.
2. Reduction of the total bases, most likely produced by the reduction of sodium, as shown by RAPAPORT.

In one case we have further thrown light upon the conditions with permanent hyperventilation. Here the reduction of the bicarbonate is completely compensated within the anion group. Hypernormal chloride values being recorded while the total bases were normal and constant here.

### Conclusion.

The experiments have illustrated how the total bases vary with physiological changes in the metabolism. The average electrolyte changes in the various groups are shown in Table 1.

Table 1.

*Electrolyte changes in connection with physiological conditions.*

	No. of Experiments	Total bases		Bicarbonate m.eq.	Chloride m.eq.	pH
		m.eq./l.	%			
Standing position .....	11	+ 2.6	+ 1.7	+ 0.9	— 0.3	
Muscular work light....	3	+ 0.9	+ 0.6	— 0.4	+ 0.5	— 0.10
heavy ..	3	+ 4.5	+ 3.0	— 12.8	+ 2.5	— 0.18
Fluid intake .....	4	— 3.6	— 2.3	+ 0.4	— 2.5	
Hyperventilation .....	4	— 1.9	— 1.2	— 4.5	+ 1.5	+ 0.16
Food consumption .....		+ 4.6	+ 3.0			

Our experiments were thus not able to verify that the total bases are kept constant during physiological conditions, to the extent stated by VAN SLYKE. The variations, within the individual groups are from 1.2 %—3 %, and may thus be of importance for the judgement of pathological values, particularly when several factors are in play simultaneously.

The cause of the variations must primarily be sought in the changes in the anion group. The changes in the anions which have been recorded are as follows: 1. Muscular work causes reduction of bicarbonate by buffer effect against the formed lactic acid. 2. The increase of respiration gives reduction of the bicarbonate (hyperventilation). 3. Increase in chloride is found in conditions with reduced bicarbonate because of the Donnan effect (muscular work, hyperventilation). 4. Changes in the base-binding capacity of the proteins occur in a) Changes in the relative protein concentration when the plasma volume is altered; b) Changes of pH in serum. Such changes occur by altered body position, muscular work, hyperventilation. 5. Intake of fluid gives reduction of chloride, presumably because of excretion of NaCl in the intestine.

These changes within the anion group lead to changes in the total anions. In order to maintain the electrolyte balance, secondary changes in the total bases occur. The physiological changes of the total bases must therefore be taken as an important compensation to maintain the electrolyte neutrality of the blood.

It must be emphasized however that all our experiments have been short-lasting. In more permanent changes in the metabolism lasting for a longer period, a more pronounced regulation presumably takes place in the anion group. The organism may then bring about other regulating mechanisms: urine secretion and the

secretion in the small intestine, as stated by GAVRILOV (1945). The referred experiment, on a condition of permanent hyperventilation, points in the same direction. Here the decrease in the bicarbonate was completely compensated in the anion group by hyperventilation chloride values, but the total bases were kept constant.

## Normal Values.

In the literature reports are available for the normal values of total bases as is shown in Table 2. As can be seen, most of the values are within 150 and 155 m.eq., but in 2 cases they are as low as 146 m.eq. Most of the materials are otherwise small and the conditions of the experiments are often not specified, and are to some extent varied in each material. Most of the investigators have used the older method with benzidine, while BROCH (1945) has applied the electrodialytic method and finds an average of 154 m.eq. for men and women.

Table 2.

*Normal values of the total bases. Earlier investigations.*

		Number
BLACKFAN (1927) .....	154 m.eq./l.	
SALVESEN (1928) .....	Men: 157, Women: 151 m.eq./l.	6 + 6
PETERS & VAN SLYKE (1931) .....	155 m.eq./l.	10
SUNDERMAN (1931) .....	146 m.eq./l.	
ATCHLEY & BENEDICT (1931) .....	152 m.eq./l.	10
HALD (1933) .....	146.5 m.eq./l.	10
BROCH (1945) .....	Men: 154.3, Women: 154.3 m.eq./l.	16 + 16

Our material consists of a total of 46 men and 10 women, all of them medical students in good health, aged between 20—35 years, the majority between 20—25 years of age, and the average age in the whole material being 23 years. It is thus a selected material. The conditions of the experiment have varied to some extent, and the material is therefore split up into various groups. There are 2 main groups: "walking" and "lying", and these are again split up into "fasting" and "non-fasting". In the latter group the experimental persons had eaten a small breakfast 2—3 hours before the samples were taken. Otherwise the following conditions were valid for all groups: 1. No food or fluid consumption had taken place the last 2 hours before the time of collecting the samples. 2. No particular muscular work had been carried out before the experiment. The persons in question had merely walked, and carried out light laboratory work. 3. All the samples were collected in the morning between 8 and 11 a. m. The



Table 3.

*Normal values of the total bases.*

	No.	Total bases m.eq./l. Mean	Standard deviation m.eq.	Standard error of mean m.eq.	Average Age years	Average Height cm.
<i>Men group.</i>						
Fasting {Lying .....	12	151.5	± 2.3	± 0.7	23	181
{Standing .....	22	152.9	± 2.6	± 0.5	22	182
Non-fasting {Lying.....	9	155.8	± 1.8	± 0.6	25	184
{Standing..	27	157.8	± 2.2	± 0.4	23	181
Total .....	70	155.0	± 3.4	± 1.0	23	182
<i>Women group.</i>						
Non-fasting, standing ..	10	157.7	± 4.0	± 1.3	21	165

blood samples were taken after a short-lasting stasis. PETERS and co-workers and BLEGEN found no change in the total bases after stasis of short duration.

The normal values in the various groups are tabulated in Table 3.<sup>1</sup> The mean values vary from 151.5 m.eq. and up to 157.8 m.eq. The lowest value being found in "lying, fasting" individuals. The highest values in "standing" individuals who 2 or 3 hours before the experiment had taken a light meal. It is again evident how the static factor effects the values with higher figures in standing position, the increase being 1.4 and 2.0 m.eq. respectively. More evident still is the effect of the meal both in "lying" and "standing" persons, the values being approx. 4 m.eq. higher. Statistically the difference between the groups is found larger than 3  $\sigma$ , in other words significant.

Apart from these effects on the metabolism, factors such as sex, age, height, weight, may also be presumed to effect the total bases. 1. *Sex*: Most of the investigations are carried out in men. The female material is small and consists only of 1 group. This shows the same average value as the corresponding group of men. The standard deviation on the other hand is slightly larger for women. The same was shown by BROCH. 2. *Age*: The material only represents young persons and the variations are too small to allow any conclusion. 3. *Height, weight*: The average height for the whole material was 182 cm., in other words above the normal as particularly tall individuals were examined. In the group "non

<sup>1</sup> Standard deviation was calculated after the formula:  $\sigma = \sqrt{\frac{\sum n^2}{n-1}}$ .

fasting, walking" a correlation coefficient  $R:0.39$  was found for the total bases and the body height. A slight correlation is in other words present (cfr. Diagram 6). Between the total base and the body surface one finds  $R:0.36$ . No such correlations are found in the other groups.



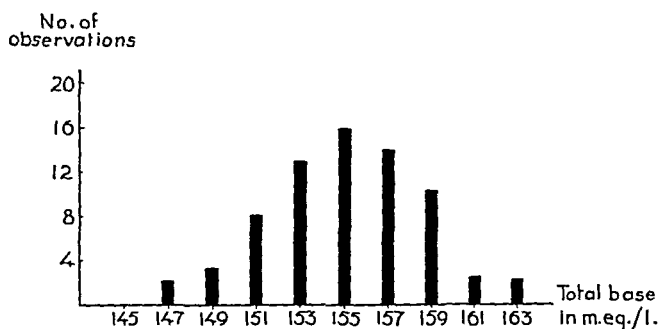
Diagram 6.

Correlation between Total bases and body height.

LANGE (1946) finds a tendency to correlation in normal persons between the protein values and the body height. The tendency to correlation between the total bases and the body height which is found in our material may therefore be presumed to stand in relation to high protein values and higher base-binding capacity in tall persons.

In the table the mean value of all experiments is stated regardless of the conditions of the experiment. We find then the same value as stated by VAN SLYKE: 155 m.eq./liter. The distribution curve for this material is given in Diagram 7. The mean figure is slightly raised however, as the number of observations of "standing" are double the number of "lying". The normal border values are when one reckons 2 standard deviations from the mean figure: 148 m.eq./liter and 162 m.eq./liter. If one reckons 3 standard deviations from the mean value one finds: 145 m.eq./liter and 165 m.eq./liter. A value of the total bases below 148 m.eq./liter

must therefore be regarded as possibly pathological, while the values cannot be regarded as certainly pathological until they are under 145 m.eq./liter. The same calculation for the group "lying" have approximately the same minimum values, while in "stand-



*Diagram 7.*

Distribution curve of the normal values for the total bases.

ing" all the values are 1—2 m.eq. higher. The lowest recorded value in our material is 146.4 m.eq./l. and the highest is 162.8 m.eq./l.

### Summary.

1. Changes in the total bases in serum are found:

a) Increase in total bases from horizontal to upright position, averaging 1.7 %.

b) Increase in total bases during muscular work, at an average of 3.5 %.

c) Increase in total bases after food consumption at an average of 3 %.

d) Reduction in total bases after fluid intake at an average of 2.3 %.

e) Reduction in total bases following hyperventilation at an average of 1.2 %.

2. The recorded changes are primarily caused by variations of the anions. When these changes are not fully compensated in the anion group the total acids are changed. Then as an important compensation, changes in the total bases occur so that the electrolyte neutrality is maintained.

3. The normal total bases values are recorded in the material consisting of 46 men and 10 women at an average of 23 years.

- a) For the total material a normal value is found of 155.0 m.eq./l. with a dispersion of approx. 10 %.
- b) In fasting persons the following is found: lying position: 151 m.eq., standing position: 153 m.eq.
- c) In non-fasting persons the following is found: lying position: 156 m.eq., standing position: 158 m.eq.
- d) The majority of the normal values are above 148 m.eq./l., but only values below 145 m.eq./l. are definitely pathological.

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From The Research Laboratories of the Radium Centre for Jutland,  
University of Aarhus, Denmark.

## **Determination by Means of Radioactive Blood Corpuscles of the Crossed Blood Flow Between Parabiotic Mice.**

By

JØRGEN BICHEL and IB HOLM-JENSEN.

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### **Introduction.**

It is a well-established fact that in animals artificially united in parabiosis substances present in the body fluids may pass from one parabion into the other. — Not only substances capable of penetrating the capillary walls, but even such substances which ordinarily are unable to penetrate the capillary walls may freely exchange between parabiotic partners.

Studying the transference of resistance to tumor transplantations between parabiotic mice the writers noticed a peculiar difference regarding the transference of immunity in homogenetic and heterogenetic parabioses. (BICHEL and HOLM-JENSEN 1947, 1948, 1949.)

If the parabions belonged to the same pure strain (homogenetic parabiosis), an induced immunity would as a rule, be transferred from one animal to the other; whereas, if the parabions were selected from different pure strains (heterogenetic parabioses), immunity was never transferred from one parabion to the other.

It is well known that all attempts to transfer immunity to tumor transplantations from one animal to another by means of blood transfusions have failed. However, the above-mentioned experiments probably demonstrate that immunity to

tumor transplantations can be transferred by humoral factors or by blood cells. It is reasonable to assume that the apparent discrepancy between the effect of parabioses and blood transfusions might be explained as a quantitative difference between the amount of antibodies transferred in the two kinds of experiments. Furthermore, it seems reasonable to investigate whether the discrepancy regarding the transference of immunity in homogenetic and heterogenetic parabioses might be considered on the same background.

In consequence of these considerations the aim of the present study has been to evaluate the rate of exchange of blood between parabiotic animals.

### Survey of Experiments and Methods.

The experimental animals were mice of the following pure strains, AKA, Do-Za, and Rf (BICHEL and HOLM-JENSEN 1949). — Both homogenetic and heterogenetic parabioses were investigated.

The parabiotic union was performed according to the technique of BUNSTER and MEYER (1933). (Muscle and skin anastomosis.)

For the determination of the rate of exchange of blood between the animals red blood corpuscles labelled by means of  $^{32}\text{P}$  were used. The labelling was carried out according to the method described for blood-volume determinations by HEVESY and ZERAHN (1942).

0.1 ml blood containing radioactive corpuscles was injected into the heart of one parabion. After the lapse of a suitable period, usually about 0.25 hour, blood samples were secured from the jugular veins of both animals. The samples were immediately centrifuged, plasma separated, and the strengths of the radioactive radiations from aliquots of the corpuscles determined by means of a Geiger-Müller counter arrangement.

In a few cases these experiments were supplemented by a corresponding measurement of the simultaneous exchange of radioactive sodium,  $^{24}\text{Na}$ .

### The Calculation of Experiments.

The labelled blood is always injected into the heart of the right parabion. This procedure lasts a few seconds, and we assume that an even distribution of the labelled corpuscles in the blood of the right animal is attained as soon as the injection is finished. We call the corresponding initial concentration of the radioactive corpuscles in the blood of the right animal,  $y_0$ . The concentration of the radioactive corpuscles being expressed in arbitrary units as the number of  $\beta$ -particles counted per minute from an aliquot of the blood corpuscles.

The concentrations of labelled corpuscles after the lapse of  $t$  hours in the blood of the right and the left parabion are denoted  $y_t$  and  $z_t$ , respectively.  $z_0$  is taken = 0.

The blood volumes of the parabions are taken as equal, and the rate of exchange of blood between the animals is supposed not to change during the experiment. Furthermore we assume for the computation that the ratio between the exchange of red corpuscles and plasma is the same as the ratio between corpuscles and plasma in the animals' blood.

Now we may calculate the rate of exchange of blood between the animals from the following equations.

$$y_t + z_t = y_0 \quad \text{I}$$

and

$$\frac{dz_t}{dt} = ky_t - kz_t \quad \text{II}$$

or

$$\frac{dy_t}{dt} = -ky_t + kz_t, \quad \text{III}$$

where  $k$  means the volume of the blood flow per time unit from one animal into the other. The unit in which  $k$  is expressed is seen to be the blood volume of one animal.

From equation I we have

$$y_t = y_0 - z_t$$

which is inserted in II in order to make integration possible, and we get

$$\frac{dz_t}{dt} = -2kz_t + ky_0. \quad \text{IV}$$

Integration gives

$$z_t = \frac{y_0}{2}(1 - e^{-2kt}) \quad \text{V}$$

In analogy with the above derivation we get from equation III

$$y_t = \frac{y_0}{2}(1 + e^{-2kt}) \quad \text{VI}$$

V solved for  $k$  gives

$$k = \frac{1}{2t} \ln \frac{y_0}{y_0 - 2z_t}, \quad \text{VII}$$

inserting I we get

$$k = \frac{1}{2t} \ln \frac{1 + \frac{z_t}{y_t}}{1 - \frac{z_t}{y_t}}, \quad \text{VIII}$$

from which equation we can figure out  $k$  by inserting the values of  $t$ ,  $z_t$  and  $y_t$  determined in our experiments.

### Details of Experiments.

About 0.5 ml blood is removed from a mouse belonging to the same strain as the individuals in which the experimental injections are to be performed. One of the carotid arteries is cut, and the blood is sampled



by means of a glass pipette in which about 0.05 ml of a 0.1 per cent solution of heparine in physiological saline has previously been introduced.

The sample is placed in a small test tube, the inside of which is coated with paraffin wax. 0.05 ml of a nearly isotonic, neutral solution of sodium phosphate containing  $^{32}\text{P}$  is added. The fluid is gently agitated and left in an incubator at  $38^\circ$  for 2 to 2.5 hours, being occasionally agitated.

After the lapse of this time at least half of the radioactive phosphorus will be found in the corpuscles.

It has been ascertained that when mixed with fresh inactive plasma after being washed with inactive saline the radioactive blood corpuscles give off less than 5 per cent of their content of radioactive phosphorus in the course of 0.25 hour, which amount for the present purpose may be neglected.

So we may take it for granted that corpuscles labelled with  $^{32}\text{P}$  are very suitable as tracers for the determination of the physiological exchange of the animals' own blood in experiments of a suitably short duration.

Before the injection of the labelled blood sample both animals are placed in a light ether anesthesia. A longitudinal incision corresponding to the sternum of the right animal is made. The injection needle which is mounted on a record syringe containing physiological saline is now introduced into the heart through the 5' or 6' intercostal space, a few millimetres to the left of the sternum, 0.05 to 0.1 ml of blood is withdrawn, the syringe is replaced by another containing about 0.1 ml labelled blood, and the injection lasting about 5 seconds is carried out. The needle is removed, a single suture is placed in the skin, and the animals are put back into their cages.

After the lapse of the experimental period, 0.2 to 0.5 hour, blood samples are secured, first from the right animal and immediately thereafter from the left. The samples are collected from carotid bleedings by means of clean glass pipettes, transferred to small test tubes covered with a rubber cap, and without delay centrifuged for 60 minutes. Then the plasma is immediately separated, and the upper layer of the corpuscles, about 3 mm, removed and discarded.

After the experiment, autopsy of the animals is performed. The pericardium of the right animal is inspected to make sure that no bleeding worth mentioning has taken place, and the skin and muscle anastomosis is in most experiments examined microscopically.

The preparation of the samples of blood corpuscles for measurement of their radioactivities is carried out as follows:

By means of a capillary pipette a small volume, in most experiments 16.5  $\mu\text{l}$ , is transferred to small aluminium dishes,  $14 \times 2.5$  mm, coated with a very thin layer of paraffin wax. 100  $\mu\text{l}$  of a 5 per cent solution of sucrose in water has previously been placed in each dish. After delivery the pipette is washed out with the sucrose solution and the corpuscles thoroughly mixed with the sucrose solution by stirring with the tip of the pipette. In this way the samples can easily be arranged

Table 1.

	Parabions					Duration of Parabiosis before Exper. Weeks	Experi- mental Period, Hours	Rate of Exchange of Blood Corpuscles 100 k
	Pair	Strain and Sex		Age Months				
		Left	Right	Left	Right			
Homogenetic Parabioses	235	AKA ♀	AKA ♀	2.3	2.3	7	0.50	31
	234	♂	♂	2.3	2.3	7	0.50	17
	371	♂	♂	4.6	4.6	8	0.25	18
	372	♂	♂	3.4	3.4	8	0.20	59
	373	♂	♂	5.4	4.4	8	0.25	44
	374	♂	♂	4.3	4.3	8	0.20	9
	224	♂	♂	2.1	2.1	22	0.50	76
	222	♂	♂	2.3	2.3	23	0.50	329
	187	♂	♂	3.4	5.1	23	0.42	360
	361	Rfl. ♀	Rfl. ♀	2.9	4.3	8	0.50	50
	363	♂	♂	2.3	2.3	8	0.50	121
	338	♂	♂	10.1	8.7	17	0.50	45
	347	♂	♂	6.8	3.7	17	0.50	17
Heterogenetic Parabioses	232	Do-Za ♂	AKA ♂	2.0	2.3	7	0.50	11
	229	♂	♂	3.0	2.3	8	0.42	1.0
	228	♂	♂	16.2	8.7	8	0.50	0.0
	227	♂	♂	1.8	2.3	22	0.50	0.0
	375	AKA ♂	Rfl. ♂	3.9	2.6	7	0.30	2.1
	376	♂	♂	2.2	2.8	7	0.25	0.0
	377	♂	♂	2.8	4.6	7	0.25	0.8
	378	♂	♂	3.0	2.8	7	0.25	0.1

The rate of exchange of blood corpuscles between parabiotic animals.

100 k means the amount of corpuscles expressed as a percentage of the number of corpuscles of one animal, which in one hour pass from one animal into the other.

so that the area of dry matter left after evaporation will be present as a central disk with a diameter of about 10 mm. It turned out to be possible to reproduce the countings of ten preparations made in this way from the same sample with a maximum deviation from the mean counting of  $\pm 3$  per cent. Samples of plasma, up to  $100\mu\text{l}$ , and in a few experiments of whole blood also were prepared in a similar way.

When evaporated to dryness at  $38^\circ\text{C}$  the samples are exposed in a Geiger-Müller counter (AMBROSEN, MADSEN, OTTESEN and ZERAHN 1945).

When possible a sufficient number of impulses are registered to bring the mean error of the countings from the individual preparations below

2 per cent, The square root mean error being computed as  $\frac{\sqrt{A + 2B}}{A} 100$

per cent, where A denotes the number of the registered impulses originating from the preparation, and B denotes the background count.

### Experimental Findings and Discussion.

The results of the experiments are given in Table 1. The rate of the exchange of blood between the parabions is expressed, as the amount of blood corpuscles which in one hour will pass from one parabion into the other, this amount being expressed as a percentage of the content of circulating red corpuscles of one animal.

The findings clearly demonstrate that the rate of exchange of blood between homogenetic parabions very considerably exceeds the rate of exchange between heterogenetic parabions, the mean rate of exchange in the homogenetic pairs being 90 % of the blood volume of one animal per hour as against 2 in the heterogenetic pairs.

This difference cannot be ascribed to agglutination of the corpuscles in the heterogenetic pairs as no agglutination phenomena can be demonstrated when mixing blood from each of the three strains in question with serum of the others.

In consequence of the present findings we have to take the rate of exchange of blood between parabiotic animals into consideration in experiments in which we fail to demonstrate any transference of immunity between parabiotic partners.

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From the Institute of Medical Chemistry, University of Helsinki.

## Effect of Sulfa Preparations and Methyl Thiouracil on the Urinary Output of Vitamin C in the Rat.

By

OLAVI KINNUNEN.

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Side by side with our increasing knowledge of the clinical properties of the sulfa preparations, an increasing amount of work is being done concerning their effect on the internal metabolism of the organism. Thus it has been shown, for instance, that they have a decisive effect on the very central process of acetylation (KINNUNEN 1946). They have also been shown to influence the vitamin economy of the organism, particularly as regards those members of the vitamin-B complex which are synthesized in the intestines (for review, cf. KINNUNEN, 1946). Furthermore, they are known to inhibit the synthesis of vitamin K in the intestines (BLACK, OVERMAN, ELVEHJEM and LINK, 1942; WELCH and WRIGHT, 1943) and to influence the excretion of vitamin E through a mechanism so far unknown (DAFT, ENDICOTT and ASBURN, 1943). On the other hand, some investigations, including my own, have shown that a lack of vitamin E, possibly caused by the sulfa preparations, scarcely has any effect on the propagation of the experimental animals or on the viability of the offspring. There have also been some references, particularly in Anglo-Saxon literature, of an interrelationship of the vitamin-C metabolism of the organism and the sulfa preparations.

Vitamin C has long been known to play a rôle in the detoxication of certain medical and toxic compounds. Earlier it was generally assumed that vitamin C is associated with the formation

of a non-poisonous coupled acid, which is excreted via the kidneys. Thus, SULZBERGER and OESER (1935), and McDONALD and JOHNSON (1941) showed that vitamin C reduces the toxicity of the (neo)-arsphenamides. The same rôle has been ascribed to vitamin C in regard to numerous other toxic compounds: germanine (v. AULER, 1935), santonine (MURAKAMI, 1939), atophan (BORSETTI, 1938), dinitrophenol (HAAS, 1939), quinine (BÜSSING, 1939), salvarsan (DAINOW, 1939), various anesthetics (DUMKE 1938), benzene (LIBOWITSKY and SEYFRIED, 1940), and various barbiturates (GREEN and MUSULIN, 1941). EKMAN (1942) showed that vitamin C influences the breakdown of certain cyclic compounds like phenol, salicylic acid, histidine, indole and tryptophan. The effect was not due, however, to a formation of coupled acids but to a rupture of the ring through the action of vitamin C, as shown later by the said author in an extensive publication (1944).

HAWLEY et al. (1936) have shown that ammonium chloride increases the *urinary output of vitamin C*. Similar observations have been made in regard to numerous other compounds: salicylates, atropine, aspirine, chinchophene, barbiturates, amidopyrine, antipyrine, adrenaline, chloroform, chloracetone, paraldehyde, stilbosterone and estradiol. It has also been found that most anesthetics increase the urinary excretion of vitamin C while, at the same time, they raise the vitamin-C content of the blood (BEYER et al., 1944). On the other hand, insulin and sodium bicarbonate seem to decrease the excretion of the vitamin (RALLI et al., 1940).

According to DAINOW (1939), ROSENTHAL (1940) and BICKEL (1940), vitamin C *reduces the toxicity of the sulfa preparations* both as regards clinical material and animal experiments. DAINOW further maintains that *the sulfa preparations decrease the urinary output of vitamin C, while the reverse effect has been observed by LONGENECKER et al. (1940), HOLMES (1943) and EKMAN (1944)*. According to HOLMES, the increasing effect of the sulfa preparations on the excretion of vitamin C is ascribable to an increased formation of coupled acids from the sulfa drugs and vitamin C or its oxidation products. EKMAN maintains that at least with rat, which is known to synthesize vitamin C, the increased excretion is due to an increased synthesis of the vitamin in the tissues. DAINOW and ZIMMEL (1939) found that the sulfa preparations decreased the vitamin-C content of the testes, suprarenal and liver of the experimental animals. On the basis of their results, DAINOW,

BICKEL and HOLMES recommend that vitamin C should be administered to patients receiving sulfa preparations.

It will be seen that there is a considerable discrepancy regarding the effect of the sulfa preparations on the vitamin-C metabolism and on the excretion of the vitamin. The author has therefore carried out a series of animal experiments in order to bring more light on the problem. Comparative animal experiments were also run in which the rats were given methyl thiouracil, as both the sulfa preparations and methyl thiouracil have been shown to possess similar metabolic influence, for instance, on the function of the thyroid. This influence has been tentatively ascribed to the changes they cause in the oxido-reductive functions of the tissues, and attempts have been made to prevent it *e. g.* by an overdosing of vitamin C (MACKENZIE, MACKENZIE and MACCOLLUM, 1941).

### Own Investigations.

The animal experiments were made with 24 adult rats of the special white strain of the Institute of Medical Chemistry. The weight of the rats at the start of the experiment was  $200\text{ g} \pm 20\text{ g}$ . The animals were divided into 6 groups of 3 each, and placed group-wise in cages equipped with funnels for collecting the urine. The urine was collected daily into erlenmeyer-flasks, which were protected against light. After the volume of the urine was measured, its vitamin-C content was determined. Daily excretion per rat in each group was obtained by dividing the total excretion by the number of rats. Prior to the actual experiment, the rats were given 2 weeks to get accustomed to their new surroundings. During this time — and throughout the experimental period as well — the animals were given ordinary standard laboratory fare *ad libitum*. Urinary output of vitamin C was followed from the beginning of the acclimatization phase.

Two groups of rats served as controls. The actual experimental period was 6 weeks. The sulfa preparations employed were: sulfanilamide, sulfapyridine and sulfathiazole. The daily dosage of the sulfa preparation in question was 50 mg per each 100 g body weight, and of methyl thiouracil 5 mg. The drugs were thoroughly mixed with the food. Changes in the body weight of the rats were noted weekly. On the 4th week of the experiment, one of the sulfanilamide-rats died, and on the 6th week one of the methyl thiouracil-rats.

Vitamin C was determined in the urine by Tillmans' dichlorophenol-indophenol method. Control work *e. g.* in this Institute had shown it to be a rapid and sufficiently reliable method (KINNUNEN and RAURAMO, 1944). The results are illustrated by the accompanying graphs.

### The Graphs Warrant the Following Conclusions.

During the 2-week acclimatization period the urinary output of vitamin C varied within fairly narrow limits.

With a daily dosage of 50 mg sulfa preparation or 5 mg methyl thiouracil per 100 g body weight, the urinary output of vitamin C was doubled or even trebled.

The increase of excretion was largest in rats receiving sulfanilamide, then followed sulfapyridine, sulfathiazole and methyl thiouracil. In regard to the sulfa drugs this order corresponds to their toxicity.

Increased excretion of vitamin C was noted during 3—4 weeks, whereupon it fell to the same level as in the control groups.

With prolonged duration of the experiment, at the end of the 6th week, the excretion was found to be lower than in the control group. This result was most marked in the sulfanilamide group.

At the conclusion of the experiment all the rats were killed and vitamin C was determined in the blood. It was found that the average values for the control group were generally slightly higher than in the experimental groups.

Table, showing the average vitamin-C content of the blood in control and experimental groups after 6 weeks, in mg%.

Control group 1	Control group 2	Sulfanilamide group	Sulfapyridine group	Sulfathiazole group	Methylthiouracil group
1.4	1.1	0.9	0.8	0.9	1.0

### Discussion.

Rat experiments showed that sulfa preparations and methyl thiouracil caused an increase of the urinary output of vitamin C during the first 2—3 weeks (cf. LONGENECKER, HOLMES and EKMAN). With the sulfa preparations this increase was proportional to their toxicity. The increased output of vitamin C is evidently associated with the detoxication of the drugs through a rupture of the ring or through the formation of coupled acids. Another possible explanation is that the synthesis of vitamin C in the tissues is increased by the stimulating action of the drugs. Detoxication obviously strains the vitamin-C synthesis in the organism and

depletes its store of the vitamin. This effected a decrease of the urinary output of the vitamin so that in some groups it ultimately fell to values below those in the control groups (cf. DAINOW). That there is an unfavourable development in the vitamin-C balance of the organism is evidenced also by the values for the

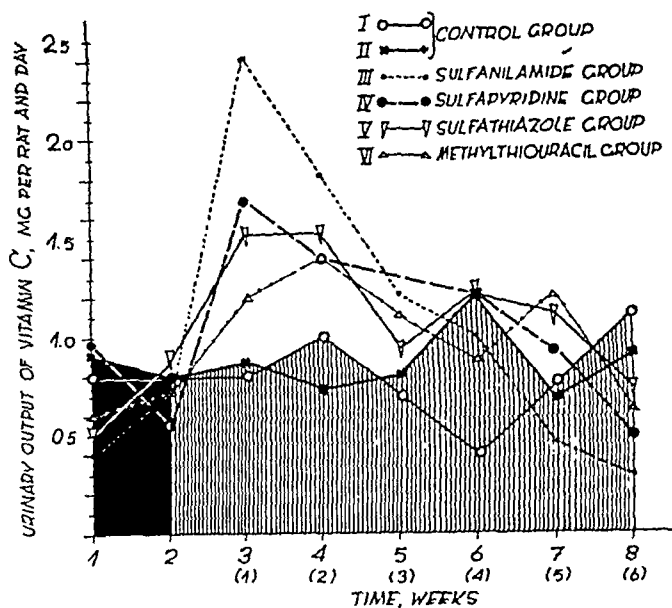


Fig. 1.

vitamin-C content of the blood at the conclusion of the experiment. They show that the values for the experimental groups, after an experimental period of 6 weeks, were distinctly lower than in the control groups.

It is interesting to compare the results of the present investigation to those of earlier workers. The results for the first 2—3 weeks agree well with those obtained by LONGENECKER, HOLMES and EKMAN, while for the later experimental period there is a good agreement with DAINOW's results. It is apparent that a discrepancy of results might arise, not only from the quality of the drug and the size of the dosage, but also from a different duration of the experiment.

### Summary.

Sulfa preparations and methyl thiouracil deplete the stores of vitamin C in the organism. In prolonged use and in large doses



they cause a lack of vitamin C which can be ascertained at least in laboratory experiments. Hence it is recommendable that vitamin C be administered in connection with the sulfa therapy especially as infectious cases themselves already cause an increased consumption of the vitamin.

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## Oxygen Consumption of Sheep Maternal and Foetal Blood.

By

M. J. KARVONEN.

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In post-natal life, a rise in the  $O_2$ -uptake of blood is recognized as indicating an increased blood formation (ITAMI 1910, MORAWITZ and ITAMI 1910). As a criterion of the rate of blood formation the  $O_2$ -uptake is to a great extent comparable with the appearance of a basophilic staining in erythrocytes (WARBURG 1909) and with a physiological reticulocytosis (HARROP 1919, RAMSEY and WARREN 1933), but no complete parallelism exists (RAMSEY and WARREN 1933).

It has been shown in various mammalian species, that the proportion of the nucleated and other "young" forms of red cells in circulation gradually decreases during foetal life, after having been very high at the early stages. It is also known, that foetal blood has a considerable oxygen uptake (FOURCROY 1790, COHNSTEIN and ZUNTZ 1884, ROESSINGH 1922). No systematic series of measurements, however, have been published on the  $O_2$ -uptake of blood during the foetal development, except a few values by CARLYLE (1946) on the blood of sheep during the last third of the foetal life. Increased  $O_2$ -uptake values have been recorded, on the other hand, also in human pregnancy — even in the absence of anaemia — but they have not been correlated to the stage of gestation (DENECKE and RÜBERG 1922).

The object of the present investigation is to furnish data on the  $O_2$ -consumption of maternal and foetal blood during the latter half of the gestation. The approach has been essentially haematological, and main attention has been directed to the

measurement of the  $O_2$ -uptake in the blood of several foetuses and of their mothers, using a method as standardized as possible. The  $O_2$ -uptake has been thus followed with the advancing foetal age and pregnancy, and material has been collected, which allows the computation of the correlation between maternal and foetal  $O_2$ -uptake values. The results are discussed, and the changes of the oxygen-uptake values are compared and correlated to the already existing information about the foetal and maternal blood.

### Material and Method.

Welsh sheep were used. The oxygen consumption of samples from 33 pregnant ewes and from 35 foetuses was measured. The foetal ages varied from 60 days to full term. In 14 maternal and in 14 foetal samples the oxygen consumption was measured also after adding methylene blue (M. B.) to make the concentration of 0.05 % M. B. in the substrate. In 28 foetal and in 28 maternal samples the haematocrit values were also determined. A morphological study of the foetal and maternal blood and bone marrow in sheep has simultaneously been carried out, to a great extent on the same material (HOOGSTRATEN and KARVONEN 1948).

The foetal samples were taken during caesarean sections from living foetuses while the placental circulation remained intact, in most cases from the carotid artery. The blood was defibrinated or — in some of the youngest foetuses — heparinized. When the effect of both the methods on the  $O_2$ -uptake was compared in a few foetal and maternal samples, no systematic difference was found, and the observed differences were within the limits of the methodical error.

After drawing, the samples were immediately put into a refrigerator, at  $+1.5^\circ C$ , and the measurements were performed within a few hours. In accordance with the results of previous investigators (RAMSEY and WARREN 1930, WRIGHT 1930) it was found, that the  $O_2$ -uptake values of blood did not change essentially, if kept in this way.

The  $O_2$ -consumption was measured at  $+38^\circ C$  using Barcroft differential manometers. 2.6 c.c. blood was used for each measurement; in some of the youngest foetuses, only 1.0 c.c. was available. The gas space of the vessels was filled with air or oxygen. A comparison of the two gases showed that this technical variation had no effect on the oxygen uptake of blood. In general, the measurements were performed according to the technique recommended by DIXON (1934). The results are expressed — when not otherwise stated — as cu.mm. oxygen used during an average minute of the first hour of incubation.

The  $Q_{O_2}$  of the blood was not constant, but decreased very rapidly on incubation. Sometimes, the logarithm of the oxygen uptake as plotted against the time of incubation was practically linear, but this relationship did not always hold. This rapid fall made it necessary to standardize the handling of the samples and the performance of the

measurements as strictly as possible. Care was taken to start the measurements immediately after temperature equilibration had been attained.

When the  $O_2$ -consumption of plasma and that of corpuscles was measured separately, a cooled centrifuge was used for the separation. The filtrations were performed in a refrigerator, using Seitz bacteriological filters.

It was ascertained in control experiments — by using the method of VAN SLYKE and NEILL (1924) — that the amount of oxygen bound reversibly by the blood was the same at the end of the temperature equilibration and after the incubation of one hour. The results, therefore, are not affected by the oxygen bound to the haemoglobin.

The measurements of haemolysis were performed by separating the plasma from the cells in a cooled centrifuge and by comparing the intensity of the colour of the plasma with that of the haemolysate of the same blood. A visual Dubosque type colorimeter was used.

## Results.

Until the 95th day all but one of the oxygen uptake values of foetal blood are between 0.19 and 0.50 cu.mm.  $O_2$  per c.c. blood. Between the 95th and 130th day the values — except two — are between 0.50 and 1.00. After the 130th day the  $O_2$ -uptake values fall again to the level which prevailed before the rise: the majority are now below the value 0.50. The changes with the age are fairly pronounced but widely scattered.

The  $O_2$ -consumption of the maternal samples is of the same order as that of the foetal samples, although on an average slightly less. ( $0.36 \pm 0.21$  for maternal instead of  $0.49 \pm 0.22$  cu.mm.  $O_2$  per c.c. blood per min. for the foetal samples.) Remarkably, the changes with the proceeding of the pregnancy are parallel with those occurring simultaneously in the foetal blood. Between the values of each foetus and its mother there is also a high and significant positive correlation:  $+0.42 \pm 0.03$ .

In order to ascertain, which component of blood was responsible for the wide variations in the oxygen uptake, the plasma or the serum and the corpuscles were separated by using the centrifuge. In some experiments, the plasma was filtered through a Seitz filter in order to get rid of all the formed elements. In one experiment, the corpuscle suspension was diluted to its initial volume by adding 0.9 % NaCl. The oxygen uptake of the blood, plasma, serum and corpuscle-suspension samples was determined separately. Table I contains the results of such measurements. They are expressed in the terms of oxygen uptake per c.c. of

Table I.

*Measurements of the O<sub>2</sub>-uptake of Plasma, Serum, Corpuscles and Whole Blood.*

Day of pregnancy	Maternal	Foetal	Sample.	O <sub>2</sub> -uptake cu.mm./c.c. sample/min.	Calculated O <sub>2</sub> -uptake cu.mm./c.c. blood/min.
99	M		Heparinized blood. Hcr: 39 % .....	0.71	
			Plasma (No haemolysis) .....	0.15	0.09
			Serum (Marked haemolysis) .....	0.13	0.08
	F		Heparinized blood. Hcr: 34 % .....	0.92	
			Plasma (No lysis) .....	0.24	0.16
			Serum (Marked lysis) .....	0.17	0.11
100	M		Heparinized blood. Hcr: 33 % .....	0.61	
			Plasma (Trace of lysis) .....	0.18	0.13
	F		Heparinized blood. Hcr: 29 % .....	0.67	
			Plasma (No lysis) .....	0.28	0.19
			Corpuscles in saline: orig. conc. ....	0.47	0.47
			Sum of the O <sub>2</sub> -uptake values of the components .....		0.66
101	M		Heparinized blood. Hcr: 30 % .....	0.56	
			Plasma, ultrafiltered (No lysis) .....	0.21	0.15
106	M		Heparinized blood. Hcr: 29 % .....	0.38	
			Plasma, ultrafiltered (No lysis) .....	0.19	0.14
	F		Heparinized blood. Hcr: 40 % .....	0.75	
			Plasma, ultrafiltered (No lysis) .....	0.28	0.17

sample, and also as calculated per the amount of the component present in 1.0 c.c. of blood. The absolute variation of the O<sub>2</sub>-uptake of the plasma occurred in these experiments within narrow limits as compared with the range of variation in the samples of whole blood. Therefore, evidently the variations in the whole blood are mainly due to variations in the oxygen uptake of the erythrocytes. This is in agreement with the findings of previous investigators. In the actual measurements, the O<sub>2</sub>-uptake of plasma or serum took a similar course as that of whole blood: there was a rapid initial rate which soon decreased.

The oxygen consumption of whole blood obviously must largely depend on the proportions in which plasma and corpuscles are present. In the following treatment, the experimental data have been used as if the contribution of the plasma would be negligible, and the O<sub>2</sub>-uptake has been calculated as being due to the corpuscle mass. As calculated in this way, the average O<sub>2</sub>-uptake values — as cu.mm. O<sub>2</sub> per c.c. packed corpuscles per minute — is for the maternal animals  $1.07 \pm 0.62$ , and for the fetuses  $1.34 \pm 0.65$ . Figs. 1 and 2 show the variations of the O<sub>2</sub>-uptake

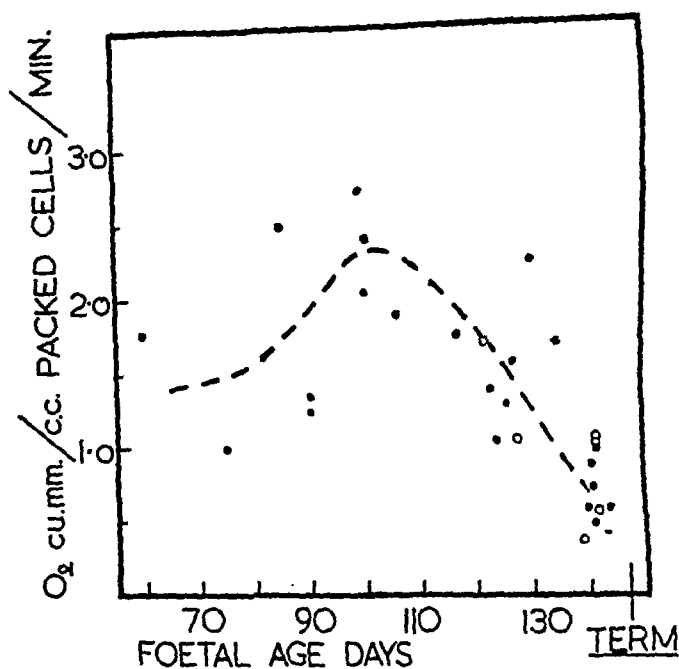


Fig. 1.  $O_2$ -uptake of foetal blood as calculated for c.c. of the packed corpuscles. The interrupted line indicates the change of the average with advancing age.

● Singlet. ○ Twin.

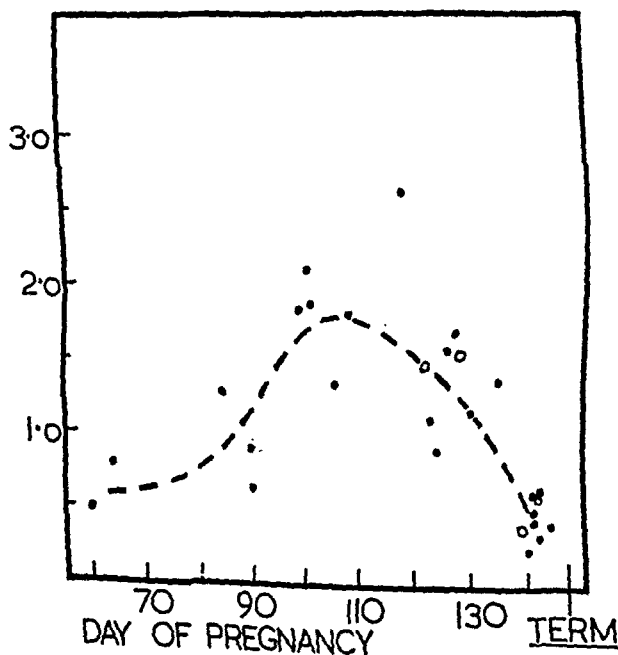


Fig. 2.  $O_2$ -uptake of maternal blood as calculated for c.c. of the packed corpuscles. The interrupted line indicates the change of the average with advancing gestation.

values per c.c. packed corpuscles as plotted against the proceeding of the gestation. The values are distributed in a less random way than the direct results of the measurements. When the correlation between the values of each foetus and its mother is calculated, a significantly higher positive correlation —  $+0.67 \pm 0.02$  — is obtained, than when using the direct results. These two facts, the more ordered distribution of the values and the higher correlation, both evidently give a justification to the approximate method of calculation used. Neither of them can be the consequence of any systematic error invented by the approximate calculation. The higher correlation of the calculated values also makes improbable a purely technical parallelism of the values simultaneously determined, because a correlation based on technical errors can be expected to be highest for the direct results of the measurements. The tugging of the ewes was arranged so that a purely seasonal variation of the oxygen-uptake values was also excluded. Accordingly, one may conclude that the correlation of the values of each foetus and its mother as well as the distribution of the values according to the advancement of gestation, both are conditioned by factors implicit in the physiological processes connected with gestation, and neither by technical nor by seasonal variations.

Incubating aseptically the samples of foetal blood for an hour at  $+38^{\circ}\text{C}$  resulted in a more or less pronounced haemolysis. GOLDBLOOM and GOTTLIEB (1929) have had a similar experience on human cord blood. Generally, the younger the foetus, the more intense the haemolysis. Under corresponding conditions, the maternal samples showed only slight lysis if any. Keeping the samples at  $+1.5^{\circ}\text{C}$ , however, protected them from haemolysis, at least for many days. On incubation, samples which were kept at  $+1.5^{\circ}\text{C}$  for 24 hours, showed the same rate of haemolysis as those incubated immediately after sampling.

After lysis, the cellular respiration becomes markedly salt sensitive (WARBURG 1911). During the actual process of lysis, great but temporary increases in the oxygen uptake have been recorded (RAMSEY and WARREN 1930, 1935). The foetal oxygen-uptake values could not, therefore, be accepted without control experiments.

Three samples of foetal and three samples of maternal blood were haemolyzed by freezing them for 24 hours at the temperature of  $-20^{\circ}\text{C}$ . The rate of the oxygen uptake was measured immediately after thawing the samples. The haemolysates showed a rapid initial oxygen uptake, but the rate soon became slower, and the values for an average minute of the first hour were not significantly different from those obtained with the non-haemolyzed samples. Whether this has any

bearing on the haemolysis which occurs during the incubation of the samples, is not known. If the incubation of the samples implies a haemolysis, it actually is meaningless to speculate about the rate of the oxygen uptake without the effect of haemolysis.

Adding M. B. in most cases had a more powerful increasing effect on the oxygen uptake of the foetal than of the maternal blood, but statistically the difference is not significant. The average oxygen uptake per c.c. packed corpuscles was for the series of investigated foetal samples  $0.83 \pm 0.30$ , and for the corresponding maternal samples  $0.53 \pm 0.34$  cu.mm. per minute. The average increase effected by adding M. B. was  $1.58 \pm 0.36$  in the foetal and  $1.16 \pm 0.77$  in the maternal samples. The catalysis by M. B. is a photochemical reaction, and the effect of it is greatly increased by light (MICHAELIS and SALOMON 1930). The amount of light was not strictly controlled in the experiments, and therefore no conclusion can be drawn about the possible changes with the advancement of gestation.

### Discussion.

The oxygen uptake values of whole blood and of erythrocytes, as obtained in the present work, are quantitatively comparable with those of other workers, in spite of considerable differences in the methods (CARLYLE: foetal sheep; ROCHE and SIEGLER-SORU 1929: adult sheep; RAMSEY and WARREN 1930: several species). Considerable species variations are known to occur, but, in normal conditions, the individual variations are relatively small (ROCHE and SIEGLER-SORU).

The values for the oxygen uptake of plasma, as given by other investigators, are considerably lower than those obtained in the course of the present work. RAMSEY and WARREN (1935) *e. g.* demonstrated an oxygen uptake in rabbit plasma, the rate of which varied between 0.02 and 0.04 cu.mm.  $O_2$  per c.c. plasma per minute. That the oxygen uptake of plasma does not entirely depend on the admixture of formed elements, has been known already since ONAKA (1911). In the present work, even the filtered samples of sheep plasma showed a quite high oxygen uptake, 0.19 to 0.28 cu.mm.  $O_2$  per c.c. per min.

RAMSEY and WARREN (1933) found that in rabbit blood oxygen uptake and reticulocyte number within certain limits moved quite parallelly, but with the progress of anaemia (produced by haemorrhage) more oxygen was consumed by the red cells than



could be accounted for on the basis that the rate of the respiration of reticulocytes and mature erythrocytes remained constant. Adult ruminants in general are known to have only very few reticulocytes in their peripheral blood. The same was shown to be true for the pregnant ewes (HOOGSTRATEN 1947). No reticulocytes were observed, and, therefore, no statement can be made as to the parallelism of the results of these two methods during pregnancy in sheep. In foetal blood the number of reticulocytes, however, allows the performance of a reticulocyte count. They show a steady fall with the advancing foetal age, paralleling the downwards slope of the oxygen-uptake values. Before the 100th day there is, however, no parallelism between these two values.

The oxygen uptake of the blood of the foetus and of its mother are of about the same order, whereas — say, at the foetal age of 100 days — the concentration of reticulocytes in the foetal blood is at least 5,000 times, probably many times more, higher than in the maternal blood. This shows conclusively that the material responsible for the reticulocyte staining has very little if anything to do with the oxygen uptake.

Whatever is the interpretation of the oxygen-uptake values of blood, the similarity of the distribution of the maternal and foetal values in the course of gestation, and the high positive correlation between the values of each foetus and its mother suggest, that the variations of the oxygen uptake in both organisms have a common cause. On the basis of an analogy to the post-natal life, to give a haematological interpretation to the oxygen-uptake values would be tempting. Accordingly, the foetal and maternal erythropoiesis would show a certain parallelism, which parallelism would be conditioned by some humoral factors, common to both organisms. However, it is premature to speculate more about the interpretations, until further quantitative data are available about the physiology of the foetal blood formation and destruction.

### Summary.

1. The "metabolic" oxygen uptake of the blood of 33 pregnant ewes and of 35 fetuses between the 60th day of gestation and the full term has been measured, by using Barcroft manometric technique.

2. The foetal samples have on an average slightly higher oxygen uptake than the samples of maternal blood. With the pro-

ceeding of gestation foetal and maternal blood show parallel changes: there is a maximum of the oxygen uptake round the 100th day of gestation, with a fall towards the term. The parallelism and the correlation between foetal and maternal values is most pronounced, if the oxygen uptake is calculated as being due to the corpuscles only.

3. The values found for the oxygen uptake of plasma are considerably higher than those reported previously in other species.

4. It is pointed out, that — on the basis of the present material — the reticulocyte staining has little if anything to do with the oxygen uptake of blood.

The author wishes to express his gratitude to the late Sir JOSEPH BARCROFT, F. R. S., who suggested the present investigation and followed its progress with a continuous interest, and to Sir LIONEL WHITBY, C. V. O., M. C., who kindly read and criticized one of the first drafts of the present paper. The author has been aided financially by a scholarship of the British Council.

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From Universitetets Biokemiske Institut, Copenhagen.

## A Further Note on Optical Specificity in Methylation Processes.

By

GUNNAR STEENSHOLT.

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In most enzymatic processes optical specificity plays an important rôle. The substrates of a large number of enzymes exhibit the remarkable property that one of the optical isomers is much more easily acted upon than the other, *i. e.*, is more readily transformed into another substance by the enzyme catalyzing the process in question. This appears to be the case also in biological methylation processes, but not much work has been done so far on this particular problem. The present writer is aware of two papers on the subject. The first is an investigation by HANDLER and BERNHEIM (1943), who studied the relative efficiencies of l- and d-methionine as methyl donators in the methylation of guanidine acetic acid to creatine. They found that d-methionine is about 50 per cent more active than l-methionine. The second contribution is a note by the present writer on optical specificity effects in the transformation of histidine and arginine into creatine in the presence of muscle tissue *in vitro* (STEENSHOLT, 1948 a). It was found that only the l-forms of these amino acids took part in the reaction. However, in the transformation histidine  $\rightarrow$  creatine it is questionable whether we are dealing with a proper methylation process (STEENSHOLT, 1948 b).

The object of the present note is to report the results of some further investigations on optical specificity effects in methylation processes in which methionine acts as methyl donator.

## Experimental Results and Their Discussion.

The biological material used in the present piece of work was rat liver tissue. It was removed from the animals immediately after decapitation, and a homogeneous pulp was prepared by finely dividing the tissue by means of a pair of scissors. Ethanol amine and dimethyl ethanol amine were both synthesised by the writer, in all essentials according to the method of KNORR (1897), and purified by repeated distillations. The dl-methionine was a Hoffmann-La Roche product. The l-methionine was presented to me by Professor M. S. DUNN, to whom I am deeply grateful for his generosity.

The work was carried out along much the same lines as our previous studies on the biosynthesis of choline. In the interest of brevity we shall therefore report in full only a few typical experiments.

One such experiment was carried out as follows: A vessel A contained 0.4 g rat liver pulp, obtained as just described, and 0.05 ml ethanol amine, suspended in 6 ml phosphate buffer ( $\text{pH} = 7.0$ ). A vessel B contained 0.4 g rat liver pulp, 0.05 ml ethanol amine and 40 mg l-methionine, suspended in 6 ml phosphate buffer ( $\text{pH} = 7.0$ ). A third vessel C was prepared in identically the same way as B, but with dl-methionine replacing the l-methionine. The vessels were incubated at  $37^\circ \text{C}$  for 6 hours. After the lapse of this period 4 ml 10 per cent trichloroacetic acid were added for the purpose of deproteinating the reaction mixtures, which, after standing for some minutes, were spun down in a centrifuge. 2 ml of the supernatant liquid were removed for the determination of choline, which was carried out in the following way. 2 ml of a saturated aqueous solution of ammonium reineckate were added, and after cooling for 20 minutes in ice water the precipitate was collected in a centrifuge tube with slender end. After centrifugation the supernatant liquid was removed by means of a capillary tube provided with a suction bulb, and the precipitate washed two or three times with ice cold alcohol. The choline reineckate was then dissolved in 1 ml acetone and transferred quantitatively to an ordinary test tube by means of some 60 per cent aqueous acetone. We then added 2 ml water, 0.2 ml 10 per cent sodium hydroxide and 0.2 ml perhydrol. The test tubes were placed in a boiling water bath, under frequent shaking. They were removed from the water bath after about 30 minutes and their contents diluted with 4 ml water. We then added 2 ml 10 per cent sulphuric acid and sufficient 0.2 per cent alcoholic solution of diphenyl carbazid to give a final concentration of 8 per cent. Duplicate analyses were of course always carried out. This is essentially the method of MARENZI and CARDINI for the determination of choline. It is based on CAZENEUVE's reaction for chromium and has previously been successfully applied to the study of methylation problems.

In the same way experiments were carried out in which ethanol amine was replaced by dimethyl ethanol amine.

The results of three experiments with ethanol amine and three experiments with dimethyl ethanol amine are summarized in Table 1. The second and third columns contain the values obtained for the relative increase in the content of choline in vessels B and C compared to vessel A, expressed in per cent. Each entry in the table is the mean of two determinations.

Similar experiments were also made at pH 6.3 and 7.9, with closely similar results, though, as was to be expected from previous results about the pH dependence of the transmethylation process under consideration here, the yields of choline were considerably smaller at these hydrogen ion concentrations, which are below resp. above the optimum value of the hydrogen ion concentration of the reaction under investigation.

Table 1.

*Relative increase in choline in per cent.*

Vessel	Series 1 pH 7.0		Series 2 pH 6.3		Series 3 pH 7.9	
	B	C	B	C	B	C
Substrate						
Ethanol amine .....	12	17	4.0	5.9	3.8	5.5
	13	17	4.5	6.3		
	12.5	17.2				
Dimethyl ethanol amine....	10	16.2	4.2	6.0	4.0	5.6
	10.5	15.5			3.8	5.5
	12	16.5				

Table 2.

*Relative increase in choline in per cent.*

Substrate	Vessel B	Vessel C
Ethanol amine .....	4.0	5.9
	4.5	6.3
Dimethyl ethanol amine .....	4.2	6.0

Table 3.

*Relative increase in choline in per cent.*

Substrate	Vessel B	Vessel C
Ethanol amine .....	3.8	5.5
Dimethyl ethanol amine .....	4.0	5.6
	3.8	5.5

It is clear from the results reported above that in the present experiments, as in those of HANDLER and BERNHEIM with guanidine acetic acid, d-methionine is considerably more efficient

than l-methionine as methyl donator. The relative efficiency of the optical isomers can be seen to be of the same order of magnitude as in the experiments with guanidine acetic acid. From previous work it is known that the processes of methylation of guanidine acetic acid and ethanol amine are closely similar in several respects. The results of the present paper, together with those of HANDLER and BERNHEIM, will probably serve to strengthen not inconsiderably the conviction that we are here dealing with enzymes or enzyme systems, which, if not actually identical, are at any rate in very close structural relation to one another.

The author is glad to express his best thanks to Prof. R. EGE for his generous hospitality.

### Summary.

A study is made of optical specificity effects in the methylation of ethanol amine and dimethyl ethanol amine to choline with methionine as methyl donator. It is found that d-methionine is considerably more efficient as methyl donator than the l-form. The significance of this fact is briefly discussed.

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From the Department of Medical Physiology, University of Copenhagen, and the Department of Physiology, University of Toronto, Canada.

## **The Effect of Fasting on Muscle Phosphorylase Activity in the Rat.**

By

**KNUD LUNDBÆK and EWALD S. GORANSON.**

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It was shown recently that the glucose uptake of isolated rat diaphragm was lower in animals maintained for some time on a carbohydrate free diet than in animals on a carbohydrate rich diet (LUNDBÆK and STEVENSON 1948). This finding, indicating a change in carbohydrate metabolism in relation to carbohydrate intake, is in agreement with many earlier observations in starvation and after reduced carbohydrate intake (LUNDBÆK 1948). The abnormalities of carbohydrate metabolism after carbohydrate deprivation have been thought to be due, at least partly, to a change in the total hormonal status of the organism. It has been shown that the insulin content of pancreas is reduced in animals on a carbohydrate poor diet (BEST, HAIST and RIDOUT 1939). The way in which the hormonal status influences the velocities and direction of enzymatic processes occurring in intermediary metabolism is only little known. One important link seems to be the hexokinase system, which has been shown to be dependent upon anterior pituitary and insulin secretion (PRICE, CORI and COLOWICK 1945). On the other hand, experiments with hypophysectomized animals seem to indicate that autonomous changes in cellular enzyme systems, independent of hormonal status, may take place in starvation or carbohydrate deprivation (FOGLIA and POTICK 1941, SAMUELS, REINECKE and BALL 1942).

In the present investigation an attempt was made to demon-

strate metabolic changes on the enzymatic level in animals which had been deprived of food for a certain length of time. Muscle phosphorylase was chosen for this purpose because of its position in the intermediary carbohydrate chain of reactions at the entrance of the glycogen stores, and above the point where extracellular carbohydrate flows into the system.

It is planned to study further the phosphorylase activity in animals maintained on a carbohydrate free diet, as well as other enzyme systems of intermediary carbohydrate metabolism, particularly hexokinase, in starvation and carbohydrate deprivation.

## Experimental.

### Methods.

Adult female albino rats, weighing between 200 and 250 grams, were used throughout in these studies. The rats were maintained on a diet of whole grain, bread, milk and water. In starvation periods water was given *ad libitum*. Re-feeding after an interval of fasting was done either by administering glucose by stomach tube or by allowing the animals access to the above mentioned diet. The fed rats were kept in cages containing food until the moment they were sacrificed. In all experiments the muscle phosphorylase activity of one fed and one starved rat were compared. In some experiments a re-fed animal was also included.

Muscle for the determination of phosphorylase activity was taken under ether anesthesia. Experiments in which sodium amytal was used in place of ether gave identical results. The procedure followed for the determination of muscle phosphorylase activity was essentially that of Cori et al. (1943). The hind leg muscles were exposed, the animals bled, and about 4—5 grams of muscle quickly excised, with as little stimulation as possible, and dropped into liquid air. The muscle was pulverized in a chilled mortar and extracted with two volumes of ice-cold distilled water. 40 minutes, with stirring, was allowed for the extraction of the protein at 0° C.

The extract was filtered in the cold, under slight suction, through coarse filter paper. The filtrate was cautiously adjusted to pH 6.1 with dilute HCl and dialyzed in cellophane tubes against running tap water (10—12° C.) for three hours.<sup>1</sup> After dialysis the extract was centrifuged in the cold to remove a small precipitate. No further adjustment in the pH of this extract was made. The extract was diluted

<sup>1</sup> Ultraviolet absorption spectra of the protein free filtrates from the dialyzed muscle extracts indicated a prevalence of inosinic acid or inosinic polyphosphate as compared to adenine nucleotide. No difference was found in the absorption spectra of filtrates obtained from the muscle extracts of fed and starved animals. We are indebted to Dr. H. M. KALOKAR for making these determinations.



with an equal volume of 0.015 M cysteine hydrochloride and kept at 0° C. until added to the reaction mixture.

The phosphorylase activity of this preparation was tested on the reversible reaction

glucose-1-phosphate  $\rightleftharpoons$  polysaccharide + inorganic phosphate both in the direction of polysaccharide synthesis and in that of glucose-1-phosphate formation from glycogen. The determinations were made both with and without addition of adenylic acid, which is necessary for the activity of phosphorylase b and enhances that of phosphorylase a (GREEN and CORI 1943).

The reaction to the right was determined by measuring the phosphate split off from glucose-1-phosphate, according to the method of CORI, CORI and GREEN (1943). The composition of the reaction mixture was as follows:

Cysteine-glycerophosphate buffer (pH 7.00) .....	1.0 ml
Glycogen, 2 % solution .....	2.0 ml
Glucose-1-phosphate, 0.064M .....	1.0 ml
Muscle extract (prepared as described) .....	1.0 ml
Adenylic acid, 0.026M .....	0.1 ml

The cysteine-glycerophosphate buffer was prepared by mixing 1 ml of 0.3 M cysteine hydrochloride with 39 ml of 1 % disodium glycerophosphate.

For the reverse reaction, namely the conversion of glycogen to the Cori ester, the same diluted muscle extract was used. Glucose-1-phosphate was omitted from the reaction mixture and 1.0 ml of 0.05M  $\text{KH}_2\text{PO}_4$  and 0.5 ml of 0.1M  $\text{MgCl}_2$  were added.

The reaction mixtures were made up in ordinary test tubes of 20 ml capacity and incubated for one hour at 31° C., with constant agitation in a Warburg bath. Following the incubation period the tubes were cooled in ice and ice-cold 10 % trichloroacetic acid added to bring the volume to 10 ml. The contents of the tubes were filtered to remove the precipitated protein and 0.5 ml taken for the estimation of inorganic phosphate which had formed or disappeared, depending on the direction of the reaction. Non-incubated tubes served as controls. A phosphate standard was set up for each experiment. Inorganic phosphate was determined by the method of FISKE and SUBBAROW (1925) using a Coleman Junior Spectrophotometer.

The protein content of the reaction mixture was determined on an aliquot of the cysteine-diluted extract by the biuret method. This was standardized against human serum, analyzed by the Kjeldahl method.

### Materials.

The glucose-1-phosphate used in these experiments was synthesized enzymatically by the method of SUMNER and SOMERS (1944), using potato phosphorylase. The glycogen was a commercial preparation (PFANSTIEL, C. P.). Adenylic acid was prepared in crystalline form from adenosine triphosphate by the method of OSTERN et al. (1938).

## Results.

The phosphorylase activity as determined by the synthesis of polysaccharide from glucose-1-phosphate in muscle extracts from fed, starved and re-fed rats are presented in Table I. In columns 2—7 of the table, values for the phosphorylase activity are expressed as micrograms of phosphate liberated from glucose-1-phosphate per milligram of protein in the reaction mixtures per hour at 31° C.

Columns 8—10 give the protein content of the reaction mixtures. No significant difference between extracts from starved and fed rats was found, the average in each case being 5.8 mg.

In columns 11—13 the phosphorylase activity without addition of adenylic acid is expressed as a percent of the activity with added adenylic acid. These percentage values, for convenience, will be referred to as the "activity ratios".

In columns 14 and 15 of the table, the relationship between the muscle phosphorylase activity values of the starved and fed rats is shown by expressing the former as a percent of the latter. These values will be referred to as the "starvation ratios".

The average activity ratio for the fed animals was found to be 33 as compared to an average value of 59 for rats which had been without food for 1 to 4 days. No correlation was found between the activity ratios of the starved rats and the duration of the fast.

The phosphorylase activity determined without addition of adenylic acid was always higher in muscle from the starved than from the fed rats as shown by the starvation ratios. After addition of adenylic acid to the reaction mixtures, however, the difference in phosphorylase activity in the fed and fasting animals was less pronounced and in a few instances no difference was observed.

The muscle phosphorylase activity of the fed and the starved rats when compared by measuring the conversion of glycogen to hexose phosphate also gave higher values for the starved animals (Table II). It thus appears that in both directions of the reversible reaction the muscle phosphorylase activity is higher in starved than in fed rats.

By a statistical analysis of the pairs of observation, the difference was found to be significant in all cases. The comparison of the

Table I.

*Muscle phosphorylase activity measured by the liberation of phosphate during polysaccharide synthesis from glucose-1-phosphate in fed (F), starved (S) and re-fed (R) rats, with (+ A.A.) and without (— A.A.) adenylic acid.*

	Liberation of P ( $\gamma$ P/mg protein)						Protein (mg) in reaction mixture			Activity ratio <sup>1</sup>			Starvation ratio <sup>2</sup>		Period of starvation (days)
	Without Adenylic acid			With Adenylic acid											
	F	S	R	F	S	R	F	S	R	F	S	R	—A.A.	+A.A.	
1	6	53		13	76		5.8	5.4		46	70		883	584	1
2	34	85		92	127		6.5	6.5		37	67		250	138	1
3	6	32		10	40		5.6	5.4		60	80		533	400	2
4	8	57	43	37	113	113	6.6	6.6	6.4	22	50	38	710	305	2
5	8	27	9	26	50	26	5.6	6.0	6.4	31	54	35	338	192	2
6	14	49		52	45		5.8	6.2		27	104		350	87	3
7	33	119		104	121		5.2	5.2		32	98		361	116	3
8	24	43		58	91		8.4	7.0		41	47		180	157	3
9	11	23	18	47	56	62	4.3	5.1	4.3	23	41	29	209	119	3
10	4	18	22	91	100	100	4.1	4.1	4.1	4	18	22	450	110	3
11	53	108	59	96	150	111	7.2	6.6	6.6	55	72	53	204	156	3
12	16	59	8	81	139	68	5.4	5.6	5.2	20	42	12	369	172	3
13	10	27	22	78	101	68	5.7	5.3	5.3	13	27	32	270	130	3
14	44	65		100	103		6.2	7.0		44	63		148	103	4
15	55	73	57	156	142	112	5.0	5.6	5.6	35	51	51	133	91	4
Mean										33	59		359	191	

<sup>1</sup> Activity without addition of adenylic acid  $\times$  100/activity with addition of adenylic acid.

<sup>2</sup> Activity in starved rats  $\times$  100/activity in fed rats.

In experiments 4, 5 and 9, 1 gram of glucose in 3 ml of water was given by stomach tube 1, 2, and 3 hours, respectively, before sampling of muscle. In experiments 10 and 11, 0.5 grams of glucose in 2 ml of water was given 3 and 3 hours, respectively, before sampling. In experiments 12, 13, and 15 the animals were re-fed the usual standard diet for 24 hours.

figures in columns 2—7 of table I gave a  $t$ -value above the 2 % limit, while the  $t$ -value for the figures of table II is very close to that limit ( $t = 3.6$ ;  $t_{02} = 3.8$ ;  $t_{05} = 2.8$ ).

The effect on the muscle phosphorylase activity of re-feeding after periods of starvation is shown in Table I. With one exception (No. 10), values for the muscle phosphorylase activity of rats re-fed for one day on the usual diet after intervals without food, or of starved rats given glucose from 1 to 3 hours before sampling the muscle were found to be lower than those of starved rats, some values approximating those of the fed animals. The activity ratios were also lower than those for the starved animals, the values ranging between 12 and 53 (average 34).

Table II.

*Muscle phosphorylase activity measured by the phosphorolysis of glycogen in fed (F) and starved (S) rats, without addition of adenylic acid.*

	Disappearance of P ( $\gamma$ P/mg protein)		Protein (mg) in reaction mixture		Starvation ratio <sup>1</sup>	Period of starvation (days)
	F	S	F	S		
1.....	8	38	4.3	5.1	475	3
2.....	27	39	6.0	6.0	144	3
3.....	14	35	5.4	5.6	250	3
4.....	3	15	7.2	6.6	500	3
5.....	11	15	6.2	7.0	136	4
Mean					301	

<sup>1</sup> see Table I.

### Discussion.

The results obtained indicate that in the resting muscle of the fed rat phosphorylase is present in both *a* and *b* forms. Since an activity ratio of 65 can be taken to represent 100 % phosphorylase *a* (CORI and GREEN 1943), the average activity ratio of 33 obtained for the fed animals denotes the presence of 51 % phosphorylase *a* in the resting muscle. The corresponding activity ratio for the phosphorylase in muscle of starved animals gave an average value of 59 representing 91 % phosphorylase *a*. This suggests that in starvation the relative proportions of the two forms of phosphorylase are altered, the *a* form which is active without addition of adenylic acid, becoming the more predominant. This process is quickly reversed when the fasting animals are re-fed, phosphorylase *a* decreasing in proportion to the *b* form.

The proportions of phosphorylase *a* and *b* are dependent upon the action of the prostetic-group removing (PR) enzyme which converts form *a* to form *b* in vitro, and which has been shown to be active in vivo during muscular activity (CORI 1945). From the results obtained it seems reasonable to conclude that the PR enzyme is active in the resting muscle of the fed rat, that its activity decreases on fasting and increases again with re-feeding.

GREEN and CORI (1943) showed that the PR enzyme is very largely removed during the process of purification and crystallization of phosphorylase *a* by adjusting the pH of the extracts to

5.8 following the primary dialysis. The results of their investigations showed that during the initial dialysis of the extracts, the enzyme exhibited only slight activity. Since in the present experiments no further adjustment of the pH was made after the initial adjustment of the extracts to pH 6.1 the PR enzyme presumably was not removed from the extracts. However, the reaction mixtures did not show PR enzyme activity during the incubation period. This was tested by comparing the phosphorylase activity of extracts from the muscles of starved and fed rats, one aliquot of each extract being prepared in the usual manner, the other adjusted to pH 5.8 after dialysis.

Adjustment of the extracts to pH 5.8 after dialysis results in a heavy isoelectric precipitation of the PR enzyme (GREEN and CORI 1943). Adjustment of the pH to 6.1 prior to dialysis produces only a very slight precipitation of protein and only when the initial pH of aqueous extracts is approximately 6.4 or higher. The pH of aqueous extracts from the muscles of starved rats was usually somewhat higher (0.1—0.4 of a pH unit) than that from the fed rats. Accordingly a slightly larger precipitate appeared during the pH adjustment. The significance of the higher pH in aqueous extracts from the muscle of the starved rats is not clear. In an experiment in which no adjustment in pH to 6.1 was made and in which the aqueous extract from the muscle of the starved rat had a higher pH than that of the fed animal, the phosphorylase activity of the former was found to be higher than that of the latter, although the difference was less pronounced than when the pH values were adjusted. An attempt to reproduce the different initial pH values by extracting samples of muscle from the same animal in water at different pH levels, subsequently bringing the pH of all extracts back to 6.1, did result in the usual difference in the small precipitates formed, but had no influence on phosphorylase activity.

A conversion of phosphorylase *b* to *a* in starvation may not be the sole explanation for the observed difference in activity of the enzyme in the fed and the starved rats. If this were so, the addition of adenylic acid to the muscle extract should elevate the phosphorylase activity of the fed rat to roughly 90 % that of the starved animals. However, the starvation ratios show that the phosphorylase activity of the starved animals as compared to that of the fed animals, while showing considerable variation, remained much higher even with addition of adenylic acid to the

reaction mixtures. This shows that the total phosphorylase activity is increased.

The possibility of an action of phosphoglucomutase or a difference in the activity of phosphoglucomutase in extracts from starved and fed animals seems excluded on account of the high dilution of the dialyzed extracts as well as from the fact that a higher phosphorylase activity was found in extracts from starved animals both in experiments where glycogen was synthesized from glucose-1-phosphate and in the experiments in which glucose-1-phosphate was formed.

The possibility that the observed differences in muscle phosphorylase activity may be due, in part, to differences in the concentration of glucose in the muscle tissue of these animals was considered. CORI et al. (1943) have shown that glucose in high concentration (0.1M) depresses the phosphorylase activity in a manner suggesting a competitive inhibition. However, no change was found in the phosphorylase activity of muscle extract to which were added concentrations of glucose calculated as the maximal difference between the tissues of starved and fed animals (0.0002M).

It seems reasonable to assume, however, that a change in phosphorylase activity in starvation is related in some way to the availability of carbohydrate in the organism. In the starved animal, where carbohydrate stores are drawn on, muscle glycogen is broken down by the action of phosphorylase. In the well fed animal the muscle is supplied by glucose from tissue fluids entering the carbohydrate cycle at a point below that at which phosphorylase plays its rôle. As seen from the re-feeding experiments an augmentation of the carbohydrate available from tissue fluids will quickly reverse the conditions towards normal. CORI (1945) has suggested that the conversion of phosphorylase *a* to *b* by the PR enzyme during muscular activity might be a regulatory mechanism which would prevent exhaustion of glycogen stores in fatigue. By the same token, the findings that in starvation the total phosphorylase activity is increased and the proportion of phosphorylase *a* is higher than in the muscles of the fed animal, may indicate a regulatory mechanism providing a more efficient mobilization of muscle glycogen when no exogenous carbohydrate is presented to the organism.

### Summary.

The resting muscle of the fed rat was found to contain a mixture of phosphorylase *a* and *b*. The total phosphorylase activity as well as the relative proportion of phosphorylase *a* was observed to increase during fasting and to decrease on re-feeding.

The change in the relative proportion of the two forms of phosphorylase suggests an alteration in the activity of the prosthetic-group removing (PR) enzyme.

The possible significance of these changes as a mechanism regulating the mobilization of muscle glycogen during starvation is discussed.

Our thanks are due to Professor E. LUNDSGAARD and to Dr. H. M. KALCKAR for valuable suggestions and criticism.

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From the Department of Physiology, University of Lund.

## Intravascular Pressure as a Factor Regulating the Tone of the Small Vessels.<sup>1</sup>

By

BJÖRN FOLKOW.

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The mechanisms involved in the regulation of vascular tone have been a matter of much controversy. Recent studies have been concerned mainly with nervous and humoral factors whereas the part played by mechanical factors have only been incidentally studied by more recent workers.

BAYLISS (1902) suggested that the blood pressure might act as a mechanical stimulus to the vascular wall, viz. the pulsatile intravascular tension should induce a certain degree of vascular tone. This hypothesis was based on a series of experiments on blood vessels and in analogy with the response of other types of smooth muscle to mechanical stimuli.

BAYLISS predominantly studied denervated limbs of dogs and cats and to some extent isolated arteries from dogs. He observed, that temporary occlusion of the femoral artery was followed by a marked dilatation of the vessels subjected to arrest of blood flow. Vasodilatation occurred even if circulatory arrest was too brief to allow any considerable accumulation of vasodilator metabolites. BAYLISS ascribes the dilator response as due to the elimination of the continuous mechanical stimulation of the vascular wall by the blood pressure. He also recorded, that denervated blood vessels, subjected to a brief and steep rise in pressure, responded by an increase in vascular tone.

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<sup>1</sup> This paper forms part of the work presented to the Medical Faculty of the University of Lund as a thesis for a Medical Degree.



BAYLISS' work was extensively criticized by ANREP (1912) who, for one thing, insisted that the vasodilatation subsequent to arrest of the blood flow, even for brief periods, was likely to be due to vasodilator metabolites. The vasoconstriction observed by BAYLISS subsequent to the rise in pressure produced by sciatic or splanchnic nerve stimulation, was interpreted by ANREP as induced by adrenaline discharged from the adrenals, the nervous control of which was unknown to BAYLISS in 1902. ANREP claimed that the calibre of blood vessels merely passively follows the variations in intravascular pressure.

WACHHOLDER (1921), studying isolated arteries, observed constrictions in the isolated carotid from the horse in response to increased intravascular pressure; these were of rather long duration and appeared with a latency of 8—30 seconds. BÜRGI (1944) made similar observations.

KLEMENSIEWICZ (1921) studied the vascular reactions to postural blood pressure variations on the denervated hind limbs of frogs. On the small vessels of the web under the microscope he observed that lowering the blood pressure elicited vasodilatation, raising the pressure caused vasoconstriction.

In his monograph "The vasomotor system" BAYLISS (1923) maintained that the marked vasodilatation subsequent to brief arterial occlusion is more likely due to the reduction of intravascular tension and not to the accumulation of vasodilator agencies. He summarizes however: "On the whole I fear that we must regard the question as undecided."

On arresting the circulation through the intestines or the hind limbs in cats for only 3—5 seconds HIROSE and SCHILF (1931) observed a subsequent vasodilatation; they thought it unlikely that chemical factors were the main cause and that BAYLISS' hypothesis offered a more acceptable explanation.

With a microscopic technique allowing direct measurements of the diameter of the pial arterioles of the cat FOG (1934) studied the reactions of the arterioli. They dilated when the general blood pressure was reduced and constricted when it was raised. The vascular reactions were of the same character whatever the cause of the variations in pressure. The vascular responses were not mediated by nervous structures, since they persisted after denervation. Further, since the arteries studied were located on the surface of the brain and the blood did not show any changes in colour, vasodilator metabolites were not thought to be engaged. More

recently FOG (1937, 1938, 1939) and FORBES, NASON and WORTMAN (1937) studied the "intrinsic" control of cerebral vascular tone. They found that local anaesthesia did not affect the vascular reactions elicited by variations of the general blood pressure, neither did denervation of the cerebral vessels that were under observation. — PORSAA (1941) made similar observations on the retinal vessels of the cat.

It is well established that a series of metabolites, including lactic acid and carbon dioxide, dilate the small vessels. Local anoxia causes vasodilatation either directly or by the release of vasodilator agents.

LEWIS (1927), summarizing his extensive studies on the blood vessels of the human skin, discusses the possible rôle of mechanical factors in vascular reactions. However, he is mainly concerned with the liberation, during the circulatory arrest, of a vasodilator agent, the H-substance, which, according to LEWIS is a normal metabolic constituent, produced in excess during anoxia and injury to the tissues.

Many authors, among them FLEISCH et al. have studied the effect of oxygen deficit and of excess carbon dioxide on the blood vessels. Carbon dioxide is recorded as having a slight but distinct vasodilator effect on the innervated perfused vessels of the dog's hind limb. Anoxia also caused vasodilatation, although hardly significant as long as the oxygen saturation of the perfusing blood exceeded 50 %. If the venous blood emerging from an adequately circulated hind limb was re-perfused into the femoral artery the venous outflow increased slightly.

FREEMAN and ZELLER (1937) brought suggestive evidence indicating that the balance between the metabolic need of the tissues and their blood supply is maintained mainly by vasodilator metabolites. They found that when the temperature of the denervated limb of a dog was raised and the metabolism thus increased, the blood flow increased sufficiently to maintain the arterio-venous oxygen difference practically unchanged. This adjustment was established during the course of a few minutes. FREEMAN (1935) observed that reactive hyperaemia following arrest of the circulation was just adequate to repay the degree of oxygen deficit as theoretically estimated from the duration of the ischaemia.

Several authors have studied the rôle of local nervous reflexes in the regulation of regional blood flow (SCHRETZENMAYR 1933,

FLEISCH 1935, MALMÉJAC et al. 1936). MALMÉJAC et al. observed that occlusion of an artery for only 2—5 seconds caused a vasodilatation in the ischaemic region, which was less in extent after cocaine intraarterially. Nicotine had no such effect. They claimed that the dilatation was potentiated by eserine and diminished by atropine.

If any local nervous mechanism of this type really exists many of the observations previously mentioned, for instance those of Bayliss, may be explained as due to the activation of this mechanism.

To summarize: although some evidence has been brought forward in support of Bayliss' myogenic theory the dominating trend among investigators is to attribute the types of vasodilatation discussed here to chemical factors and to local nervous mechanisms.

In a study on reactive hyperaemia in cats and dogs FOLKOW, HAEGER and KAHLSON (1948) observed incidentally that, when the blood flow through the hind limbs was arrested because of extensive vasoconstriction a brief occlusion of the abdominal aorta elicited a conspicuous vasodilatation. Since in this actual experiment the vasodilator response could hardly be due to vasodilator metabolites these investigators concluded that mechanical or nervous mechanisms, activated by the unloading of the small vessels from the pulsatile arterial pressure, might be involved.

The subject of the present study is to investigate the validity of the myogenic theory as originally proposed by BAYLISS. A myogenic mechanism, if existing, might be an integral factor in the regulation of vascular tone, as well as in the first phase of vasodilatation in reactive hyperaemia following arterial obstruction.

## Experimental.

### Part I. The Possible Rôle of Local Nervous Mechanisms.

MALMÉJAC et al., already referred to, postulated that a local nervous mechanism is engaged in inducing the vasodilatation evoked by a brief arterial occlusion. They concluded that synapses are not involved. Since the general opinion is that there are no ganglionic cells around the minor blood vessels, nervous structures, if such really are engaged, are likely to be of the axone type nerve fibers belonging to spinal or possibly sympathetic neurons. A series of experiments were carried out to investigate this point.

*Method.* The experiments were made on cats anaesthetized with chloralose, 60 mg/kg or chloralose-urethane 40 mg + 400 mg/kg. The animals were eviscerated, leaving the kidneys and the liver with their arterial supply intact. The abdominal aorta was dissected and all branches except those supplying the hind limbs tied. The inferior caval vein was exposed and all subsidiaries draining tissues other than the legs were ligated. The spinal medulla was crushed from L IV downwards and the spinal channel plugged with cotton wool. The abdominal sympathetic chains, if not removed in a previous operation, were extirpated. In order to exclude all vascular connections, except the abdominal aorta and the inferior caval vein, between the upper and lower part of the animal, the body wall was mass-ligated at the height of the fifth lumbar vertebra. In some of the experiments this rather tedious procedure was omitted, without any noticeable influence on the results. To prevent clotting heparine was injected intravenously. Blood pressure was recorded from the brachial artery by a mercury manometer. In some of the experiments the blood pressure was concomitantly recorded in the hypogastric artery. The venous outflow from the hind limbs was recorded by the device described by GADDUM (1929). A collecting cannula was inserted in the distal stump of the caval vein, from where the blood was led to the recorder. From this it was collected in a funnel which served as a small venous reservoir. This funnel was connected with the cannulated proximal stump of the caval vein and thus the blood was directed back to the animal. Vasodilatation was evoked by brief obstruction of the abdominal aorta. Concomitantly the venous return from the reservoir was discontinued by tightening a clamp.

### Results.

1. *Experiments on hind limbs with intact vasomotor supply.* Arrest of the blood flow for as short a period as 3—5 seconds sufficed to evoke a slight but significant vasodilatation. With prolongation of the period of arrest vasodilatation became more pronounced, which is in full accord with what is well established for reactive hyperaemia. Since the increase in flow occurred simultaneously with the steep fall of blood pressure, the increase in the rate of flow must be due to vasodilatation, predominantly in the arterioles. If the period of obstruction was shorter than 1—2 minutes the dilatation was of short duration (10—25 seconds). (Fig. 1 A.)

2. *Experiments on animals with acutely denervated vessels.* After sympathectomy or total denervation of the hind limbs temporary arrest of the blood flow still elicited vasodilatation of principally the same type as in intact animals. Because of abolished vaso-constrictor tone after denervation the resting blood flow through

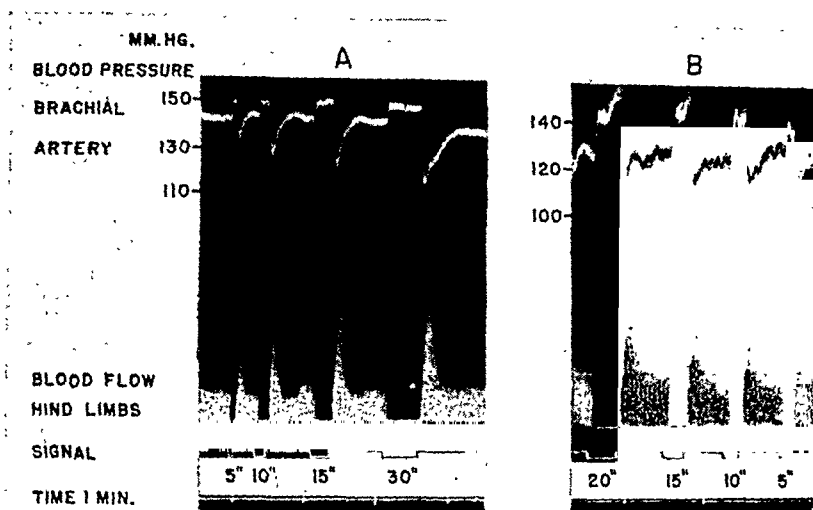


Fig. 1. A. Cat 3.8 kg. Chloralose. Intact vasomotor innervation. Vasodilatation following obstructions of arterial supply.

B. Cat 4.5 kg. Chloralose. Abdominal sympathetic chains extirpated. Vasodilatation following obstruction of arterial supply.

From above: Blood pressure, blood flow through the hind limbs, signal, time mark.

the hind limbs was increased. Consequently the vasodilator response was less conspicuous, although it could be easily observed, even after occlusions of short duration (3—5 seconds). (Fig. 1 B.)

3. *Experiments on animals with the hind limbs sympathectomized by previous operation.* In order to exclude the participation of local sympathetic chains the hind limbs were sympathectomized

by extirpation of the abdominal sympathetic chains in five cats. The post-ganglionic fibres were allowed to degenerate for about three weeks before the actual experiment. The vasodilatation subsequent to arrest of the blood flow was principally identical with the vasodilator response observed in the two previously reported groups. Fig. 2 illustrates vasodilator responses to obstructions during various periods of time from 2—10 sec.

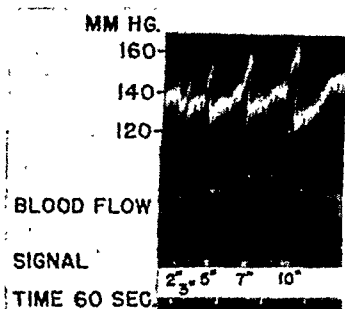


Fig. 2. Cat, 3 kg. Chloralose. Abdominal sympathetic chains extirpated 3 weeks earlier. Vasodilatation following obstructions of arterial supply.

From above: Blood pressure, blood flow through the hind limbs, signal, time mark.

4. *Experiments on animals with one hind limb de-afferented.* To exclude the possibility that somatic axone reflexes play a part in this type of

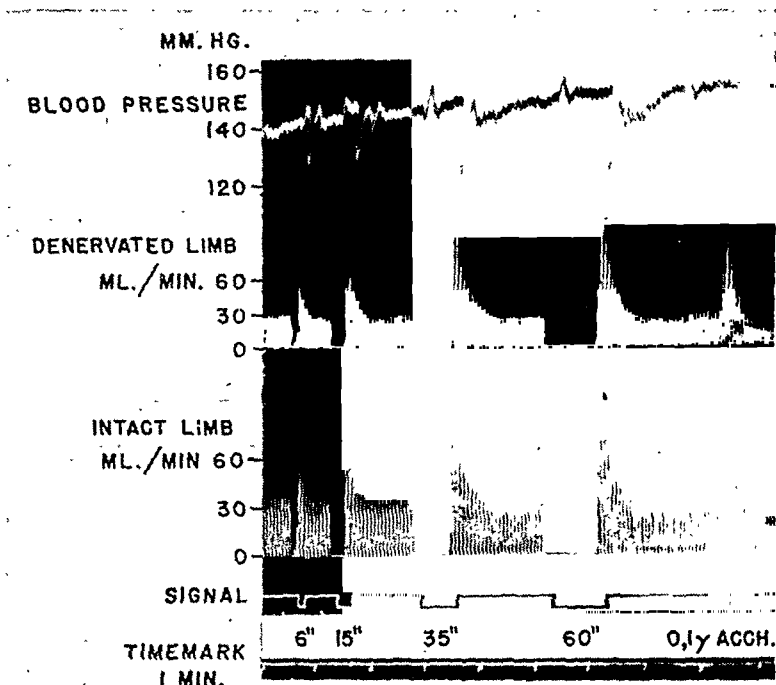


Fig. 3. Cat, 3.5 kg. Chloralose. Dorsal root fibres supplying the left hind limb allowed to degenerate. The blood flow is separately registrated from the both hind limbs. Vasodilatations following obstructions of arterial supply.

*From above:* Blood pressure, blood flow through the left hind limb, blood flow through right hind limb, signal, time mark.

vasodilatation in seven cats the spinal ganglions belonging to all lumbar and the three upper sacral segments were removed on the left side. The afferent fibres were then allowed to degenerate for three weeks. In the actual experiment the blood flow in each hind limb was recorded separately. The inflow-cannulas to each of the two Gaddum recorders were inserted into the common iliac vein of each limb. As seen in figure 3 there was no significant difference between the vasodilator response in the normally innervated and the denervated hind limb.

#### Comments.

The postulate of MALMÉJAC and HAIMOVICI (1936) and others that local nervous mechanisms are involved in the vasodilatation following arterial obstructions of short duration is not supported by the present experiments. The vasodilator response is not significantly altered by denervation and consequent degeneration of the nerve fibres to the vessels. Contrary to the report of MAL-

MÉJAC et al. atropine which was administered in many of the present experiments did not reduce the vasodilator responses. The observations by these authors that cocaine intraarterially abolishes this type of vasodilatation does not justify the conclusion that nervous mechanisms are engaged. The diminished response after cocaine might as well be due to a depressed sensitivity of the smooth muscles of the vascular wall to mechanical or chemical stimuli.

The present experiments do not allow any conclusions as to the validity of the experiments by FLEISCH and SCHRETZENMAYR on "ascending nutritional reflexes" supposed to influence the tone of larger arteries. The technique used in the present paper predominantly records variations in arteriolar tone, and minute variations in the tone of larger arteries will probably not be revealed. Since FLEISCH was unable to demonstrate the activation of "ascending nutrition reflexes" in reactive hyperaemia following occlusion of the arterial supply, the involvement of a nervous mechanism of this kind in the regulation of arteriolar tone is rather improbable.

## Part II. Experiments Supporting Bayliss' Myogenic Theory.

*Method.* Experiments were performed on 36 cats, 5 dogs and 3 rabbits. The animals were anaesthetized with chloralose, chloralose-urethane or nembutal. The choice of anaesthetic did not significantly influence the vascular reactions observed. However, since under chloralose the animals generally showed a better general condition and exhibited a higher vascular tone, this anaesthetic was usually used.

In the majority of the experiments the animals were artificially respired. Usually they were slightly hyperventilated.

1. *Vascular response to decreased intravascular tension.* These experiments were performed on the hind limbs, a few on the skin and some on the gut.

In experiments on the hind limbs the technique was principally the same as described in Part I. The blood flow from the caval vein was registered by a Gaddum recorder and the blood pressure measured from the brachial artery. The arterial inflow pressure exerted on the vessels of the hind limbs was reduced by partial or total occlusion of the abdominal aorta at the height

of the fifth lumbar vertebra. The blood pressure in the aorta below the level of obstruction was measured from a cannula inserted in the hypogastric artery. In this way the blood-pressure variations in the hind limbs produced by aortic obstruction were continuously recorded by a mercury manometer.

To record the blood flow through the skin a cannula was inserted into a saphenal vein and connected with a drop recorder of the "Ordinatenschreiber" type. This technique is fully described in a paper by CLEMENTZ and RYBERG (1949) included in this volume. Where Gaddum's recorder is used the height of the ordinates are directly proportional; with the "Ordinatenschreiber", inversely proportional to the rate of flow.

In seven cats experiments were made on the intestinal vessels. The abdomen was opened by a midline incision and the spleen removed. The splanchnic nerves were cut just below the diaphragm. The superior mesenteric artery was dissected at its origin from the aorta and stripped of its adjacent nervous plexus for a distance of a few millimeters. Cranial and caudal vascular connections from the other splanchnic regions were tied by ligatures around the proximal end of the duodenum and around the ileocecal region. The superior mesenteric vein was dissected. In such preparations the small intestines were supplied with blood solely from the superior mesenteric artery and drained by the mesenteric vein, the vessels being deprived of all central-nervous influence. The kidneys were then removed and the pressure in the intestinal arteries recorded from a cannula inserted in the abdominal aorta just distal to the superior mesenteric artery. The systemic blood pressure was measured in the brachial artery. Variation of intestinal blood pressure was effected by partial occlusion of the abdominal aorta just proximal to the origin of the coeliac and superior mesenteric arteries. The superior mesenteric vein was attached to a Gaddum recorder and the blood from the reservoir directed back to the inferior caval vein or the portal vein of the animal.

Experiments on the intestinal vessels met with some difficulties as the inevitable rather crude manipulations of the intestines frequently reduced the sensitivity of the vessels to any type of stimulus.

The preparation generally was in a good condition with a satisfactory vascular tone for one or two hours. To avoid drainage from the circulation, the funnel at the beginning of the experiment was filled with either heparinized blood from another animal or



with an 8 % solution in Tyrode of "Dextran" (GRÖNWALL and INGELMAN 1944).

2. *Vascular response to increased intravascular tension.* The vessels were also exposed to increased intravascular tension. The observations were made on the denervated hind limbs of cats. The animals were prepared as previously described. The intravascular tension was increased by raising the general blood pressure. This had to reach a certain magnitude with a rather steep ascent and descent of pressure changes in order to reveal a constrictor response in the denervated vessels. A steep increase of the blood pressure was obtained by occlusion of both common carotids. This caused a rise of pressure due to reflex vasoconstriction in the upper half of the body, which had an intact vasomotor supply. In order to eliminate depressor reflexes the vagi were cut. To exclude interference of reflexly discharged adrenaline the adrenals were removed.

To permit proper observations the animals had to be kept in good condition, under light anaesthesia and with adequate respiration. Animals subjected to blood losses or too deep anaesthesia or otherwise in bad condition showed only weak vascular responses, and sometimes they were almost entirely abolished. — It should also be stressed that artificial perfusion of an isolated denervated region, a technique used in many previous investigations on vascular reactions, renders the vessels so insensitive to various kinds of stimuli, that this technique seems less helpful in studying more delicate vascular reactions. In perfused isolated hind limbs of cats the vascular responses to brief arterial obstruction or to vasodilator drugs were, in my hands, considerably reduced or even abolished.

### Results.

#### 1. *Vascular response to decreased intravascular tension.*

a) *Hind limbs (muscular vessels).* Observations were made on more than 25 cats. The blood pressure was systematically reduced as to degree and duration. In all experiments the vessels responded to a reduction in pressure with a dilatation. The vasodilatation was evident from the slow increase of flow that occurred during the course of persisting arterial obstruction, provided that the obstruction could be maintained at a constant level, as well as from the abrupt increase in flow and the consequent fall of blood pressure that ensued when the occlusion was discontinued. The gradual increase of blood flow during a period of partial arterial occlusion

is evident from figure 4. This figure as well as figure 5 show the relationship between the degree of reduction of blood pressure in the hypogastric artery (representing the inflow pressure to the hind limbs) and the magnitude of vasodilator response. Fig. 5 demonstrates the influence of the duration of arterial obstruction on the vasodilator response. It will be seen that the vasodilator response increases with increased reduction in intravascular tension, as well as with the duration of the obstruction. The period of obstruction requisite to induce a maximal increase of blood flow following a pressure reduction of a certain degree varied considerably in different experiments, from 20 to 60 seconds. In the experiment seen in figure 5 the maximal vasodilator response occurred when the obstruction was maintained for 25 seconds.

From observations on about 25 cats with intact sympathetic supply to the hind limbs the rate of circulation in these regions was calculated to 2.5—4.5 ml./min./100 g tissue. After sympathectomy as well as after total denervation the blood flow in the hind limbs was approximately doubled. In the experiment shown in figure 5 the total blood flow in the hind limbs amounted to 30—40 ml

before sympathectomy (3.5—4.5 ml./min./100 g tissue). After extirpation of the abdominal sympathetic chains the rate of blood flow increased to about twice this figure (7.5 ml/min. in figure 5). The venous outflow from the sympathectomized hind limbs usually was almost as bright red as the arterial blood. During the application of short periods of partial arterial obstruction the bright red colour of the venous outflow from the

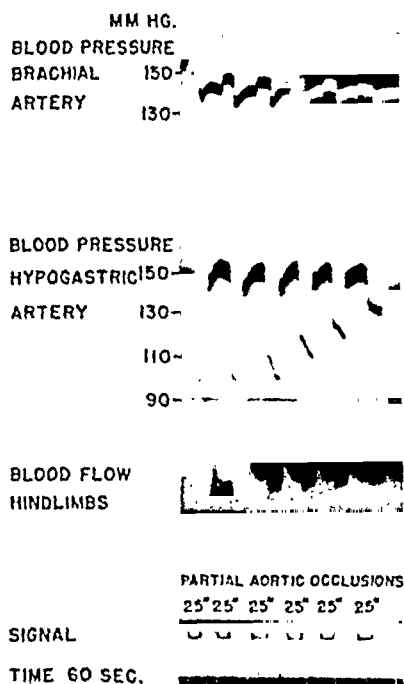


Fig. 4. Cat 3 kg. Chloralose. Artificial respiration. Spinal medulla crushed from L IV downwards, the abdominal sympathetic chains extirpated. Partial obstructions of the abdominal aorta. The increase of flow during the partial obstruction is especially evident in this figure.

From above: Blood pressure (brachial artery), inflow blood pressure to the hind limbs (hypogastric artery), blood flow through the hind limbs, signal, time mark.

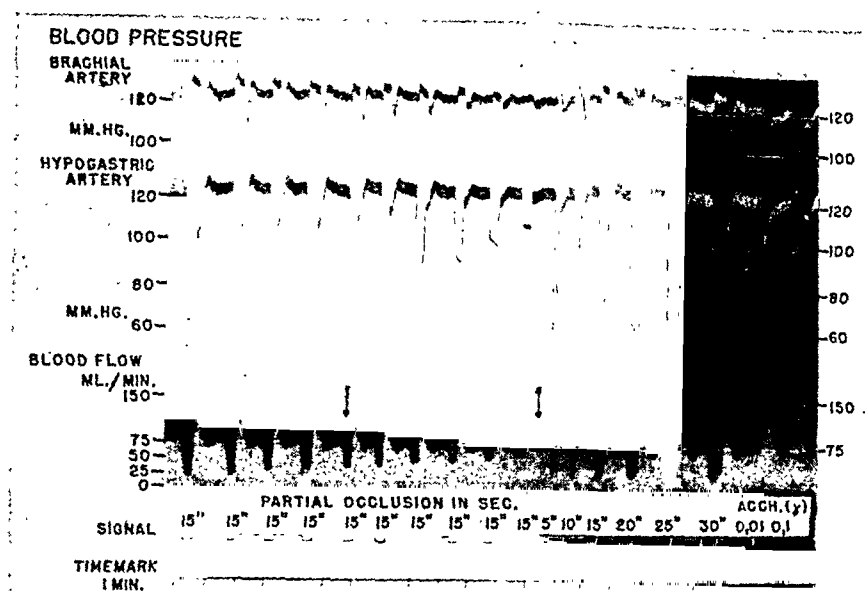


Fig. 5. Cat 4 kg. Chloralose. Same performance as in fig. 4. Partial obstructions of the abdominal aorta to varying degrees and duration.

From above: Blood pressure (brachial artery), inflow blood pressure to the hind limbs (hypogastric artery), blood flow through the hind limbs, signal, time mark.

hind limbs did not darken perceptibly, indicating that the blood flow during restriction of the circulation was not reduced to a degree where the nutrition of the tissues became inadequate. In fact, marked vasodilator responses were observed even when, during the period of partial arterial obstruction, the blood flow was reduced to a level above that prevailing before sympathectomy (represented by the situation between arrows in figure 5). — In order to exclude the interference of adrenaline released from the adrenals these were extirpated or denervated in seven experiments. Their removal did not influence the vascular reactions concerned.

Similar experiments were performed on the hind limbs of 5 dogs and 3 rabbits. The results were principally identical with those obtained in cats. Observations on rabbits met with some difficulties as it was hard to maintain these animals in good condition. They frequently did not resist the necessary operative procedure.

#### b) Cutaneous vessels.

Observations were made on 6 cats. The skin vessels showed the same type of dilator response to decrease of intravascular pressure as seen in the muscles, although vasodilatation in the skin was less pronounced. Fig. 6 A shows an experiment where muscular

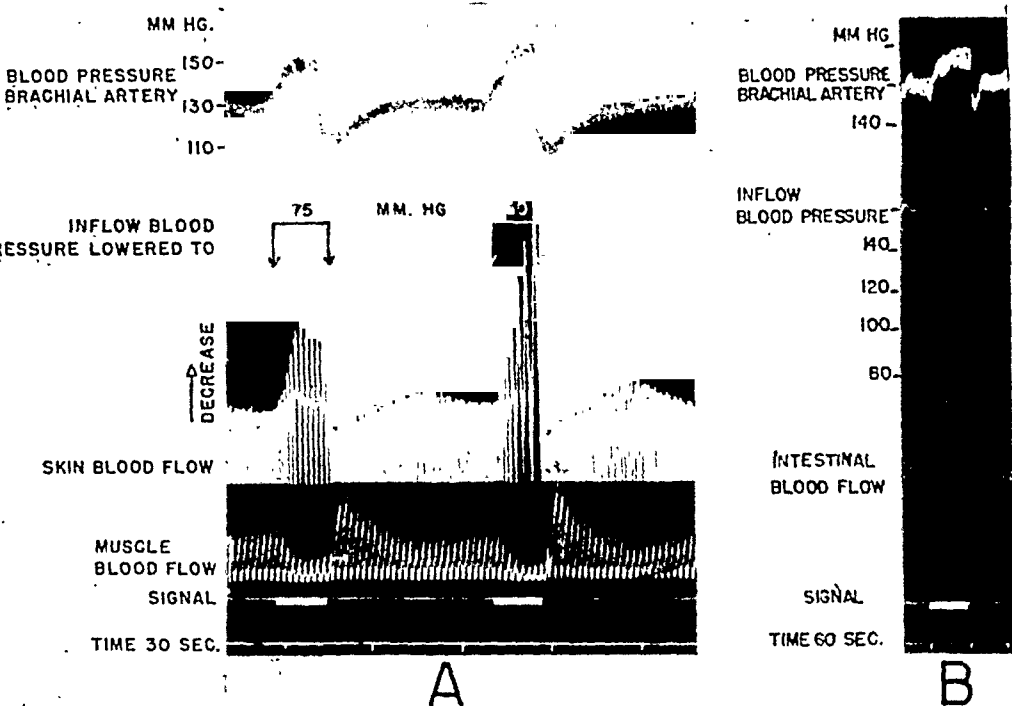


Fig. 6. A. Cat 3 kg. Same performance as in fig. 4. Caval outflow and saphenal outflow registered separately. Partial obstruction of the abdominal aorta.

From above: Blood pressure (brachial artery), notation of inflow blood pressure, skin blood flow, muscular blood flow, signal, time mark.

B. Cat 4 kg. Chloralose. Intestinal blood flow. Partial occlusion of the supplying artery.

From above: Blood pressure (brachial artery), inflow blood pressure intestinal blood flow, signal, time mark.

and cutaneous blood flow are recorded concomitantly. Reduction of the blood pressure induces a vasodilatation in the cutaneous as well as in the muscular vessels. (Note the different technique of recording the blood flow from the caval and saphenal veins. The caval blood flow is recorded with a Gaddum recorder and consequently the height of the ordinates is proportional to the magnitude of the blood flow. The cutaneous blood flow is registered with an "Ordinatenschreiber". Thus the ordinates here are inversely proportional to the magnitude of the blood flow. An increased skin blood flow will be registered as decreased ordinates.)

#### c) Intestinal vessels.

Observations were made on seven cats. One of the experiments is presented in figure 6 B. The vascular reactions to diminished intravascular tension were principally identical with those of the

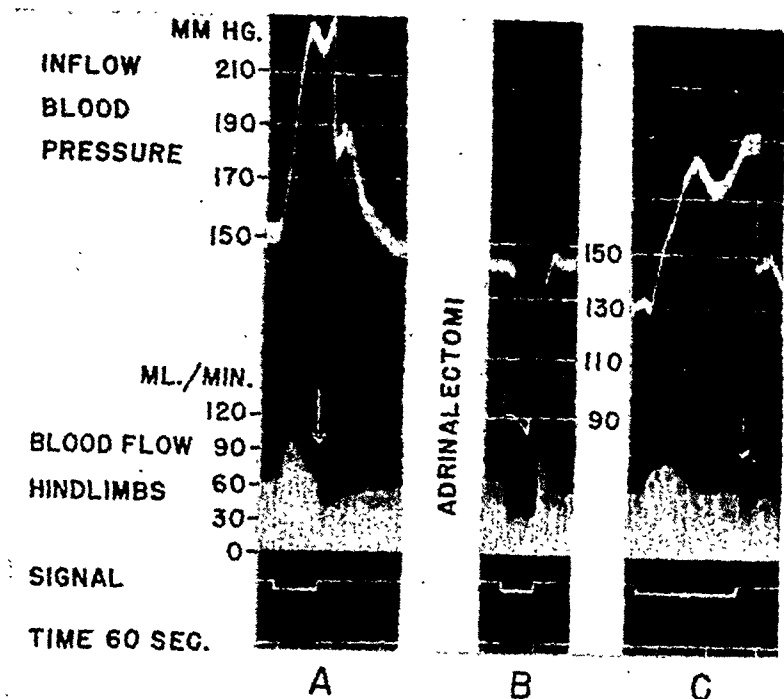


Fig. 7. Cat 3.8 kg. Chloralose. Artificial respiration. Hind limbs denervated. Adrenals extirpated between "A" and "B". Vagi cut. Blood flow through the hind limbs registrated.

A. Occlusion of common carotids; adrenals intact.

B. Inflow pressure lowered 35 % for 40 seconds. (Adrenals extirpated.)

C. Occlusion of common carotids, 40 % increase of inflow pressure. Note the increase of flow at the beginning due to increased perfusion pressure, which is soon followed by a constriction of the vessels in the hind limbs. At arrow this constriction is unmasked when blood pressure rapidly returns to its original level.

From above: Inflow blood pressure (hypogastric artery), blood flow through the hind limbs, signal, time mark.

muscular and cutaneous vessels, although they usually were less pronounced.

## 2: Response to increased intravascular tension.

Observations were made on six cats. The hind limbs were denervated and the adrenals extirpated. The general blood pressure was abruptly raised by occlusion of both common carotids in the neck. Due to the greater pressure head the rate of flow through the hind limbs increased, but after a few seconds the flow gradually decreased although blood pressure was maintained at the high level. When, on unclamping the carotids the blood pressure dropped steeply to the initial level, the caval blood flow diminished below the original level for a short period thus unmasking the

constriction of the vessels. The fact that the rate of flow does not passively follow the variations in blood pressure indicates vasoconstriction in the denervated vessels of the hind limbs. This is illustrated in fig. 7. At mark A the adrenals are intact. Just before mark B the adrenals were extirpated. The unmasked vasoconstrictor response following the period of increased pressure (indicated by ↓ in figure) still persists at mark C. Thus, the constrictor response appears at least partly to be due to the increased intravascular tension. The more pronounced and protracted constriction seen at mark A might be due to the reflex release of adrenaline from the adrenals. For comparison the dilator reaction to a slight decrease in intravascular tension is shown at mark B. In some of the experiments it proved difficult to unmask the constrictor response shown at ↓ in figure 7, because the fall in blood pressure on unclamping the carotids was too gradual. However, it was quite evident from these experiments also that increased blood pressure induced vasoconstriction in the

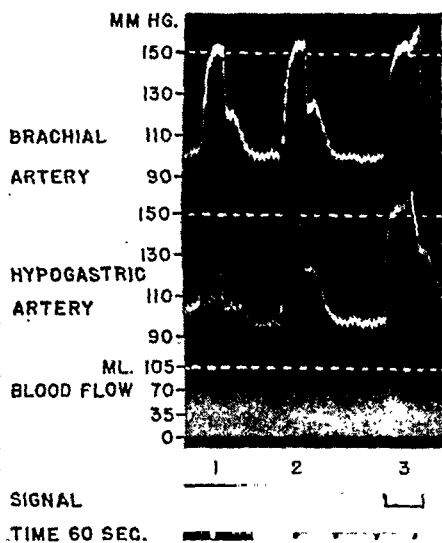


Fig. 8. Cat 3.5 kg. Chloralose. Same performance as in fig. 6. Adrenals extirpated.

1. Occlusion of common carotids. By partial occlusion of the abdominal aorta the blood pressure is kept practically unchanged in the hind limbs.

2. 3. Occlusion of common carotids without any prevention of the pressure rise in the vessels of the hind limbs. 50 % increase of pressure but only a slight transient increase of blood flow.

denervated vessels of the hind limbs. In the experiment presented in fig. 8 the blood pressure was raised from 100 to 150 mm Hg, representing an increase of the perfusing pressure of 50 %. If other factors remain unchanged, blood flow is directly proportional to the pressure, and the rate of flow should increase at least 50 %. As seen in the figure the increase of blood pressure is associated with only a slight transient increase of flow. After 5—10 seconds the flow declines to and remains at the initial level, in spite of the persisting high inflow-pressure. This indicates a vasoconstriction in the hind limbs. That the vasoconstrictor response is not due to humoral agents is seen at the section marked 1 in figure 8. At this point the

rise of intravascular pressure in the hind limbs is prevented by partial occlusion of the abdominal aorta. Under these circumstances the blood flow through the hind limbs is not significantly reduced in response to a rise in systematic pressure evoked by clamping the carotids. In the majority of these control experiments no decrease in flow was observed when the blood pressure was raised in the fore part of the animal. Only in a few experiments a minute reduction of the venous outflow occurred; this effect was, however, too small to have any significant bearing on the increase of tone in the vessels exposed to heightened intravascular tension.

In some experiments it was impossible to obtain any significant vasoconstrictor responses of the denervated vessels to increase of blood pressure. The vessels appeared to be almost indifferent to variations of intravascular tension. In such cases it also proved difficult to obtain the dilator response to decrease of intravascular pressure. As a rule typical vascular reactions in denervated vessels were observed only if the animal was in a good condition.

#### Comments.

The experiments show that total denervation of muscular, cutaneous and splanchnic vessels does not abolish the vascular reactions of these vessels to variations in intravascular tension. There are no indications that the vascular reactions of denervated vessels, observed after partial obstruction of the arterial inflow, are due to accumulation of  $\text{CO}_2$  or reduction in oxygen tension. The rate of blood flow through the denervated hind limbs was often higher during the period of partial arterial occlusion than with free circulation before denervation. This fact seems to exclude the possibility that arterial obstruction to this limited degree caused an inadequate blood supply in the limbs with consequent major alterations in the tension of oxygen and carbon dioxide. The possibility remains that partial obstruction of the blood flow causes the accumulation of vasodilator substances, such as Lewis' H-substance. The possible intervention of chemical agents in the observed vascular reactions will be discussed in the next chapter.

The present observations are in accord with those of FOG (1934, 1937, 1938, 1939) and FORBES et al. (1937) who recorded that the cerebral vessels respond to heightened intravascular tension with a constriction and to lowered tension with dilatation. As nervous and chemical factors seemed to be ruled out, they concluded that the reactions observed could be satisfactorily explained in line

with BAYLISS' myogenic theory. PORSAA (1941) studying the retinal vessels arrived at the same conclusion.

### Part III. Chemical Factors.

A series of experiments was performed to assess the possible rôle of vasodilator metabolites in the vascular response evoked by restriction of blood pressure.

*Method:* The experiments were made on 14 cats under chloralose. The animals were eviscerated, the hind limbs denervated and the blood flow measured as previously described. In all experiments the adrenals were either extirpated, or denervated by cutting the splanchnic nerves.

1. In one series of experiments the concentration of  $O_2$  and  $CO_2$  in the arterial blood was varied. The lungs were artificially ventilated with a mixture containing various concentrations of oxygen and carbon dioxide. To avoid spontaneous respiratory movements the animals were slightly hyperventilated. The oxygen concentration in the mixture varied from 8 % to 100 %, the carbon dioxide concentration from 0 to 5 %.

2. In a second series of experiments the effect of diminished flow in the hind limbs, produced by increased venous pressure, was compared with the effect of a similar reduction of flow, produced by diminishing the arterial inflow-pressure. In these experiments the animal was divided into an upper and a lower part by mass ligatures at the height of the fifth lumbar vertebra, and the vessels in the spinal channel obstructed by a tampon. In this preparation the caval vein formed the only venous drainage of the hind limbs. The increased venous pressure was obtained by partial obstruction of the caval vein.

3. In a third series of experiments the vascular effect of intra-arterial injection of venous blood was studied.

### Results.

1. Fig. 9 is from an experiment where the oxygen and  $CO_2$  concentration of arterial blood is varied. At mark A the animal is ventilated with 100 % oxygen, at B with a mixture containing 95 %  $O_2$  and 5 %  $CO_2$  and at C with a mixture containing 8 % oxygen and 92 %  $N_2$ . No considerable change occurs in the blood flow through the denervated hind limbs when the oxygen saturation of arterial blood is being diminished from 100 % to about



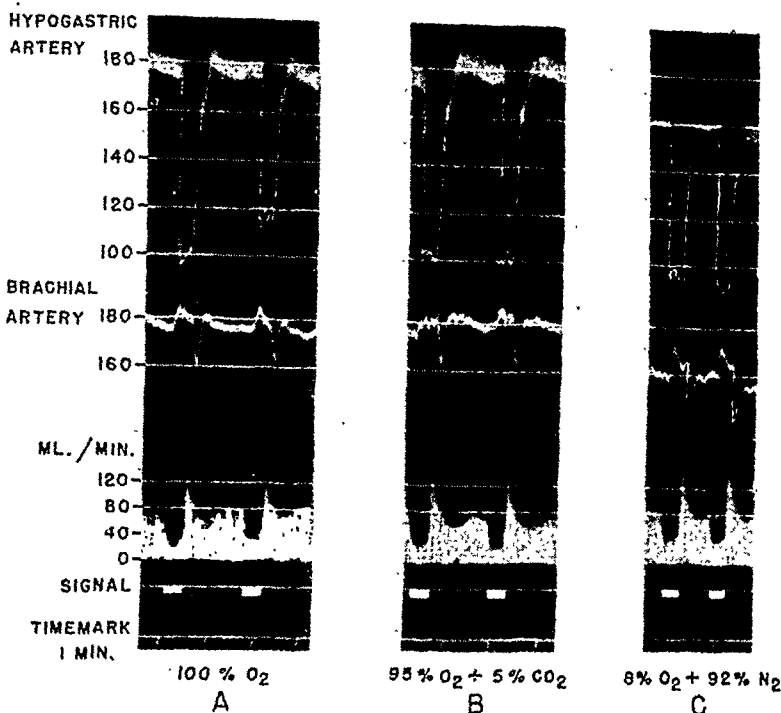


Fig. 9. Cat 4.2 kg. Chloralose. Artificial respiration. Hind limbs denervated, adrenals extirpated. Vasodilator responses to partial occlusions of the abdominal aorta.

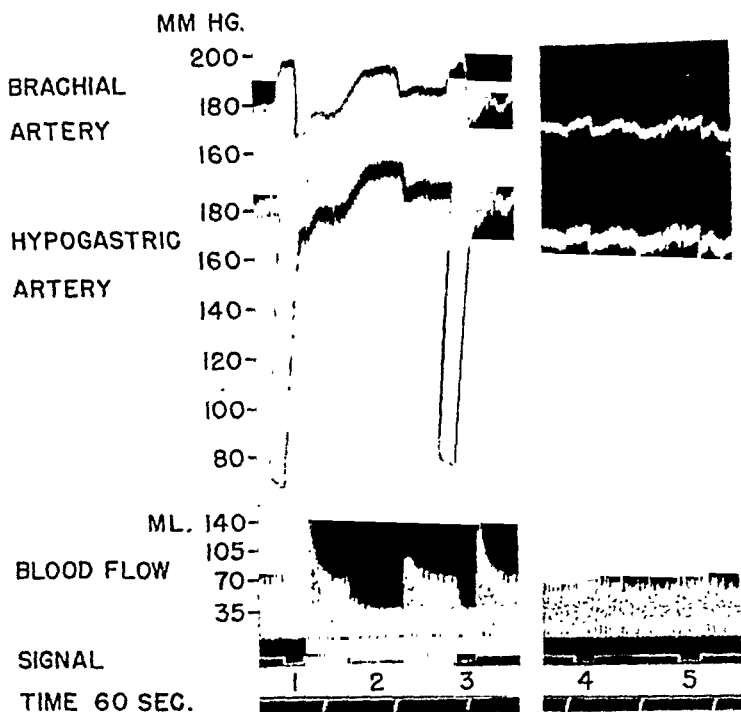
A. The animal ventilated with 100 %  $O_2$ .

B. (4 minutes after A) The animal ventilated with a mixture of 95 % and 5 %  $CO_2$  since 3 minutes.

C. (4 minutes after B) The animal ventilated with 8 %  $O_2$  and 92 %  $N_2$  since 3 minutes.

50 % and the arterial tension of  $CO_2$  can be expected to be more than doubled. On the other hand, the blood flow increases by 60–80 % on reduction by about 50 % of the intravascular pressure in the hind limbs for a short period. It is hard to conceive, that the relative ischaemia and subsequent oxygen want and increase in  $CO_2$ -tension produced in the hind limbs by this limited reduction of flow, should be more pronounced than the state induced by ventilating the animal with the gas mixtures mentioned above.

2. In the experiment illustrated in fig. 10 the rate of blood flow through the hind limbs was reduced by obstruction of the abdominal aorta or the caval vein. Release of the arterial occlusion was followed by a pronounced increase of the caval outflow and a fall of blood pressure of about 15 mm Hg. On the other hand, release of the venous obstruction, maintained for 50 seconds, in-



1. 3: PARTIAL ARTERIAL OCCLUSION 15 SEC.  
 2: PARTIAL OUTFLOW OBSTRUCTION 50 SEC.  
 4. 5: INJECTION OF 10 ML. VENOUS BLOOD  
 INTO INFERIOR MESENTERIC ARTERY

Fig. 10. Cat 4 kg. Chloralose-urethane. The animal divided by mass ligatures at the height of L V. The spinal medulla crushed from L IV downwards, the spinal channel obstructed. Influence on caval outflow of arterial and venous obstruction and of intraarterial injections of venous blood.

From above: Blood pressure (brachial artery), inflow blood pressure (hypogastric artery), blood flow through the hind limbs, signal, time mark.

duced only a slight increase in caval outflow without any significant fall of blood pressure.

The fact that arterial occlusion evoked vasodilatation is evident from the concomitant increase of flow and fall of blood pressure. The changes associated with venous obstruction are more difficult to interpret. During the period of raised venous pressure, blood will accumulate in the capillaries and the veins. When the venous obstruction is released, emptying of the overloaded vessels will cause a transient increase of venous outflow. Accumulation of vasodilator metabolites, as a result of venous obstruction, must be considered a possibility in these experiments. Whatever the inter-

pretation, the observation that the increase of flow subsequent to arterial occlusion is of much greater magnitude than the increase following venous obstruction, strongly opposes the possibility that chemical agents are predominant in vasodilatation induced by partial arterial occlusion of short duration.

3. Intraarterial re-injection of venous blood into the cannulated stump of the inferior mesenteric artery did not produce any considerable vasodilatation; the blood was withdrawn from the hind limbs during the state of free circulation. This is shown in fig. 10 where 10 ml venous blood was injected during the course of 15 seconds.

### Comments.

These experiments do not support the view held by LEWIS and others that vasodilator metabolites are the only factors responsible for arteriolar dilatation following arterial obstruction. Decrease of intravascular tension appears to be potent in causing relaxation of the vascular smooth muscles.

### General Comments.

The present study has been mainly concerned with the factors involved in eliciting the type of vasodilatation observed subsequent to brief occlusion of the arterial blood supply. Principally three mechanisms may play a rôle in this type of vasodilatation: activity in local nervous structures, vasodilator metabolites and finally mechanical factors provided by changes in blood pressure.

The experiments of this report have been so directed as to exclude the interference of the two first mentioned factors as probable causes of the vascular responses observed. It has been demonstrated that mechanical factors, *i. e.* changes in the degree of stretching of the vessels, effected by the blood pressure, are efficient in causing changes in the tone of the small blood vessels.

From the experiments of this study it is not clear whether reduction in the pulsatile pressure changes or the fall in the head of mean pressure constitutes the predominant stimulus for the vascular wall to relax. Partial obstruction of an artery causes changes in both these factors.

It seems premature to try to assess more definitely the rôle of this mechanism in integrated peripheral vascular regulation.

However, it is near at hand to assume, that the degree of intravascular tension, respectively changes in tension, constitute stimuli instrumental in maintaining and modifying vascular tone.

These observations and the interpretation given in the present paper are in accord with recent findings of BOZLER (1947) on the response of smooth muscle to stretch. Recording local action potentials BOZLER observed that sudden distension of a region of the ureter with pressure above threshold produces a local response like that preceding spontaneous contractions. This also applies to a continuous distension which elicited constant discharge. These observations give additional support for the myogenic origin of the peristaltic contractions. The mechanical stimuli effected by stretch seem to be a predominant factor in maintaining and modifying tone and activity in smooth muscles of diverse origin.

### Summary.

Observations are described which indicate that the permanent stretching of the vascular smooth muscles, effected by the blood pressure, constitutes a stimulus which elicits increased tone in the small blood vessels. Reduction, respectively elimination of this stimulus is thought to be a major factor in the vasodilatation occurring in the first stage of reactive hyperaemia.

Part of the expenses for this investigation was defrayed by a grant from the Medical Faculty of the University of Lund.

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From the Department of Physiology, University of Lund.

## The Vasodilator Action of Adenosine Triphosphate.

By

BJÖRN FOLKOW.

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Adenosine compounds have been credited to dilate the coronaries (DRURY and SZENT-GYÖRGYI 1929—30, BENNET and DRURY 1931 and GILLESPIE 1934), the muscular vessels (FLEISCH and WEGER 1937, SCHOEDEL 1935, KALCKAR and LOWRY 1947), the intestinal vessels (MARCOU 1932, DE WAELE and v. D. VELDE 1945) and the cutaneous vessels (BENNET and DRURY 1931). In lower concentrations adenosine compounds are reported to dilate the pulmonary vessels (GADDUM and HOLZ 1933), and to constrict them in high concentrations (GADDUM and HOLZ 1933, EMMELIN and FELDBERG 1948).

The relative dilator potency of the different adenosine compounds has not been quantitatively established. According to FLEISCH and WEGER (1937) the dilator action of adenosine triphosphate (ATP) is 100—150 times stronger than that of adenylic acid. According to GILLESPIE (1934), SCHOEDEL (1935) and KALCKAR and LOWRY (1947) the compounds are approximately equal in potency, while DRURY (1932) states that the activity increases somewhat with phosphorylation.

Adenosine compounds have a wide distribution in the body, especially striated muscles contain large amounts, predominantly as ATP. Because of its wide distribution and high biological activity, it has been suggested that ATP might play a rôle in peripheral vascular regulation.

In most of the previous experiments large amounts of adenosine compounds were given to produce vasodilator effects, from 50—

100  $\gamma$  up to many mg were generally administered. SCHOEDEL (1935) and FLEISCH and WEGER (1937) observed vasodilatation in the hind limb of dogs on intraarterial injection of ATP calculated to give a concentration in the blood of approximately 1:1400000.

FOLKOW, HAEGER and KAHN (1948) observed that intra-arterial injection of 0.1  $\gamma$  ATP was sufficient to produce a significant vasodilatation in the hind limbs of the cat. They stressed that ATP possesses potentialities as a normal vasodilator metabolite, a suggestion previously made by BILDINGS and MAEGRAITH (1937) and others. RIGLER (1932) brought some evidence to indicate that ATP or other adenosine compounds play a rôle in the local regulation of blood flow during muscular activity.

It is the purpose of the present study to investigate the vasodilator action of ATP in various regions.

### Method.

The experiments were performed on cats and dogs narcotized with chloralose (70 mg/kg), chloralose-urethane (50 mg + 500 mg/kg) or nembuthal (30 mg/kg).

A. *Muscular and cutaneous vessels of the hind limbs in cats.* The animals were eviscerated. The blood flow was separately recorded in the abdominal caval vein and in one of the saphenal veins. The caval blood flow was directed to a GADDUM recorder (GADDUM 1929). The flow from the saphenal vein was registered by a drop recorder. Intraarterial injections of ATP were made in the cannulated stump of the inferior mesenteric artery. Intravenous injections were made in the cannulated brachial vein. Some of the experiments were performed on animals with their hind limbs sympathectomized or de-afferented 3 weeks previously.

B. *Intestinal vessels.* These experiments were performed on cats. The technique has been described by FOLKOW, FROST and UVNÄS (1948). The outflow from the superior mesenteric vein was measured with a Gaddum recorder and the blood pressure was registered from a brachial artery. Intraarterial injections were made into the rubber tubing inserted in the divided superior mesenteric artery.

C. *Coronary vessels.* These experiments were performed on dogs with a cross circulation technique previously described by FOLKOW, FROST and UVNÄS (1948). The coronaries of the fibrillating heart of a recipient dog were perfused from a donor dog. The outflow from the coronary sinus was measured with a Gaddum recorder. ATP was given intra-arterially into the rubber tubing connecting the carotid artery of the donor dog with the perfused coronaries of the recipient.

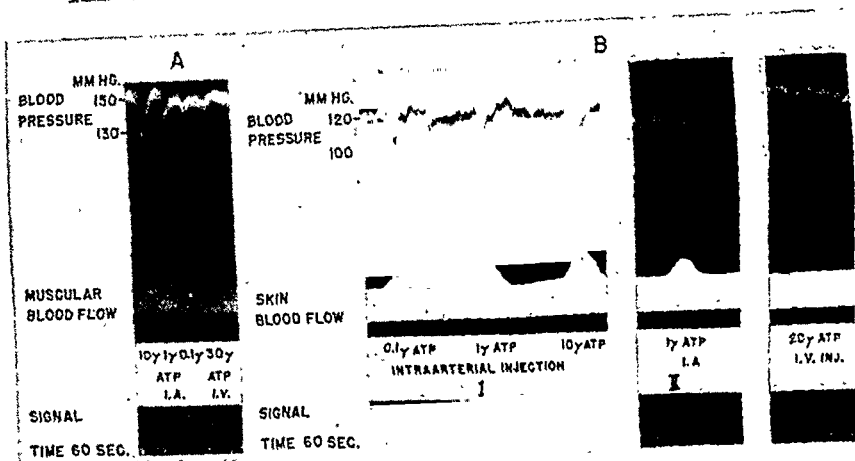


Fig. 1.

A. Cat, 3.5 kg. Chloralose-urethane. Atropine 0.1 mg/kg given. Intraarterial and intravenous application of ATP.

From above: Blood pressure, blood flow through the hind limbs, signal, time mark.

B. Cat 4 kg. Chloralose-urethane. Registration of skin blood flow. The volume of the flow is calculated from the number of drops registered per minute. Intraarterial and intravenous injections of ATP. At "II" 1  $\gamma$  ATP is mixed with blood and injected 2 minutes later. Compare the action with "I".

From above: Blood pressure, skin blood flow, signal, time mark.

## Results.

ATP was observed to elicit a pronounced vasodilatation in all vascular regions studied. The threshold-dose for the vasodilator effect amounted to 0.05–0.1  $\gamma$ . Total denervation and consequent degeneration of the vasomotor nerves did not significantly influence the vasodilator responses to ATP. Neither atropine nor neoantergan, given in doses sufficient to completely abolish the vasodilator action of moderate doses of acetylcholine or histamine, antagonized the vasodilator action of ATP. Quantitatively, expressed in equimolar concentrations, the vasodilator potency of ATP amounted to about 1/5–1/15 of the potency of acetylcholine.

Fig. 1 illustrates the vasodilator action of ATP on muscular and cutaneous vessels. Intraarterial injections of 0.1–10  $\gamma$  elicits a pronounced vasodilatation. The figure further illustrates the remarkable fact, that intravenous injection of ATP in amounts exceeding the threshold dilator dose 200–300 times does not exert any significant depressor action. Contrastingly acetylcholine and histamine cause pronounced depressor responses when given intravenously, even in minute amounts. Evidently ATP is rapidly destroyed in the circulation. This destruction does not appear to



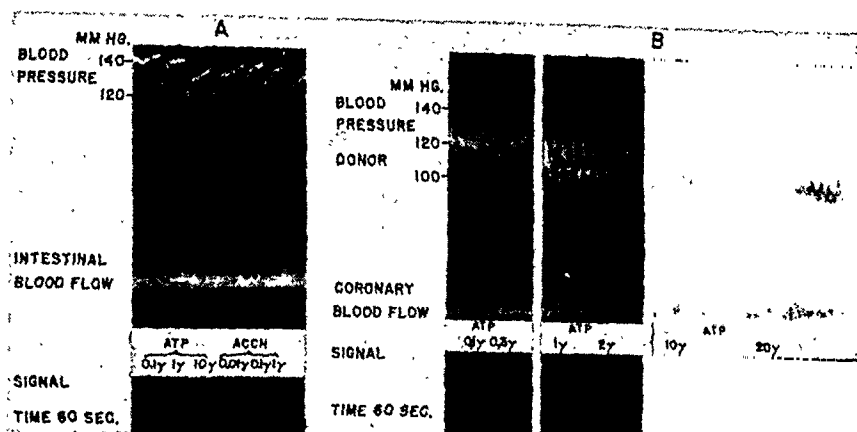


Fig. 2.

A. Cat, 3.5 kg. Chloralose-urethane. Blood flow through the intestines. Intra-arterial injections of ATP and acetylcholine.

From above: Blood pressure, intestinal blood flow, signal, time mark.

B. Dogs, cross circulation. Donor 11 kg, recipient 7 kg. Nembutal. Atropine 0.1 mg/kg is given. Intraarterial injections of ATP.

From above: Blood pressure donor, coronary blood flow of recipient, signal, time mark.

occur in the blood. At mark II in fig. 1 1  $\gamma$  ATP is mixed with 1 ml blood and the mixture allowed to stand for 2 minutes in 37 °C. When injected intraarterially the dilator response to ATP remains unchanged as compared with mark I in figure 1. Fig. 2 A shows the vasodilator effects of ATP and acetylcholine on the intestinal vessels of a cat. It is difficult to compare the dilator action of ATP and acetylcholine on the intestinal vessels, since even small amounts of acetylcholine cause rather strong contractions of the intestines. These contractions interfere with the circulation mechanically by temporarily increasing the vascular peripheral resistance. The amounts of ATP used in these experiments (not more than 20  $\gamma$ ) had no perceptible action on the intestinal smooth muscles. Therefore it seems as if the increase of flow following administration of ATP is due to a pure vasodilator action.

In fig. 2 B the dilator effect of ATP on the coronaries of a dog is shown. Amounts of ATP from 0.1—20  $\gamma$  were observed to induce a marked dilatation. The effect was not influenced by administration of atropine. No significant action on the heart muscle was observed by these minute amounts.

Intravenous application of ATP in amounts of 10—30  $\gamma$  were never with certainty observed to have any effects on respiration or heart rate.

### Discussion.

ATP proved to be a very potent dilator substance in all vascular regions investigated. Its dilator potency is comparable to that of acetylcholine and histamine. It was previously observed (Folkow, HAEGER and KAHLSON 1948) that the dilator action of ATP was not blocked by atropine or neoantergan. This observation which is confirmed in the present experiments indicates that the dilator action of ATP is not produced by the release of any of these potent dilator agents. Nor are any nervous structures engaged since the dilator action of ATP remains approximately unchanged after denervation of the vessels.

It was previously stressed (Folkow, HAEGER and KAHLSON 1948) that recording the arterial blood pressure alone is a very poor indicator of vasodilator responses. This is especially true when ATP is given intravenously, since the substance seems to be rapidly destroyed in the lungs. Further, when given in larger amounts ATP constricts the pulmonary vessels and induces a reflex bradycardia (EMMELIN and FELDBERG 1948). The fall in blood pressure observed when ATP is injected will then at least partly be due to these mechanisms and the peripheral dilator action difficult to observe.

Neither can the dilator action of ATP occurring in intact animals be quantitatively estimated in perfusion experiments as the artificial perfusion considerably reduces the vasodilator responses of the perfused vessels.

RIGLER (1932) concludes from experiments on frogs that adenosine compounds might play a part in eliciting local vasodilatation in contracting muscles. BILDINGS and MAEGRAITH (1937) claim that similar substances appear in the venous blood on the release of arterial ischaemia. There is some evidence to indicate that adenosine compounds are released from traumatized tissues (BENNET and DRURY 1931). Further GREEN (1943) showed that most symptoms seen in the crush injury syndrome could be closely imitated by injections of muscle extracts, the active agent of which seemed to be ATP. It seems desirable to investigate more closely the possible rôle of ATP in peripheral vascular regulation.

### Summary.

ATP is a potent vasodilator agent, the minimal active doses amounting to 1/5—1/15 of those of acetylcholine when calculated

as equimolar concentrations. The vasodilator action seems to be due to a direct action on the vessels. On intravenous injection ATP is rapidly destroyed, presumably in the lungs.

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From the Department of Physiology, University of Lund.

## Cutaneous Vasodilatation Elicited by Local Heating of the Anterior Hypothalamus in Cats and Dogs.

By

BJÖRN FOLKOW, GUNNAR STRÖM and BÖRJE UVNÄS.

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The earlier literature concerning the central nervous control of body temperature has been extensively reviewed by THAUER (1939) and by RANSON (1940). In the present paper reference is limited to only a few papers of special interest in connection with the present investigation.

In the intact animal an increase of body temperature activates a coordinate heat loss mechanism including polypnea or panting, cutaneous vasodilatation and sweating. Experimentally the nervous mechanisms concerned in regulation of heat loss can be activated by heating of hypothalamic structures. MAGOUN *et al.* (1938) observed panting and sweating as the result of strictly localized heat stimulation of the preoptic and supraoptic regions in cats under urethane anaesthesia. By the use of the Horsley-Clarke instrument two electrodes were inserted in the hypothalamus and the structures between the tips of the electrodes heated by a high frequency alternating current. By a subtemporal approach HEMINGWAY *et al.* 1940 placed small gold foil electrodes on the hypothalamus of dogs. Heating the anterior part of the hypothalamus of an unanaesthetized animal by diathermic current caused inhibition of shivering and a cutaneous vasodilatation, recorded as a rise of temperature of the ear. Panting did not occur in these experiments.

During the course of an experimental study in our laboratory

on the efferent nervous pathways involved in cutaneous vasodilatation, cutaneous vasodilator responses were elicited by local heating of the hypothalamus. As the technique used offered possibilities of furnishing additional facts concerning the functional interrelation between hypothalamus and the cutaneous circulation the following experiments were made.

### Method.

The experiments were performed on cats and dogs under nembuthal (30—50 mg/kg i. v. or i. p.), chloralose (50—70 mg/kg i. v.) or urethane (1 g/kg i. v.).

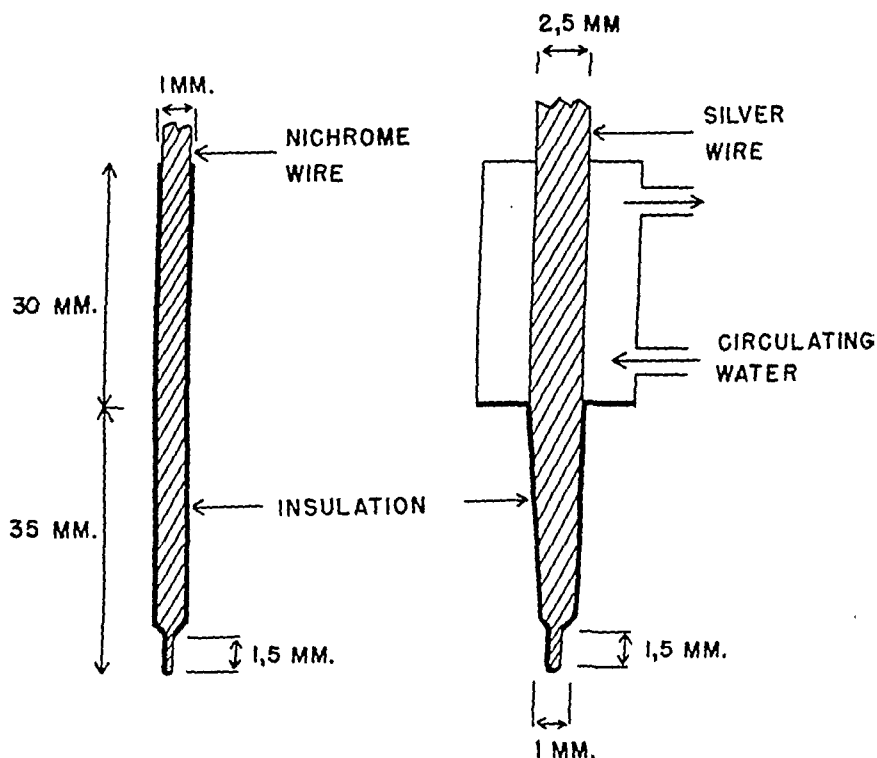


Fig. 1. Schematic drawing of the two types of electrodes used for diathermic heating and conduction heating.

The anterior part of the hypothalamus was heated locally by the use of a pair of parallel electrodes oriented with the HORSLEY-CLARKE technique. The skull was opened and the electrodes inserted in a frontal plane symmetrically to the sagittal midline through small incisions in the dura. (RANSON 1940.)

The distance between the electrodes was 5—6 mm. Two types of electrodes were used. One type consisted of nichrome wire insulated

to within 1 mm from the electrode tip (MAGOUN et al. 1938). The other type was made of silver wire and specially constructed to allow conduction heating or cooling by circulating water (see fig. 1). In addition heat could be produced by a diathermic current passing between the uninsulated tips of the electrodes.

The diathermic current was produced by an apparatus delivering high alternating current with a frequency of 1 megacycle per second. The apparatus consisted of a line operated electrone-coupled oscillator and a power amplifier with an intake of about 4 Watts. In order to

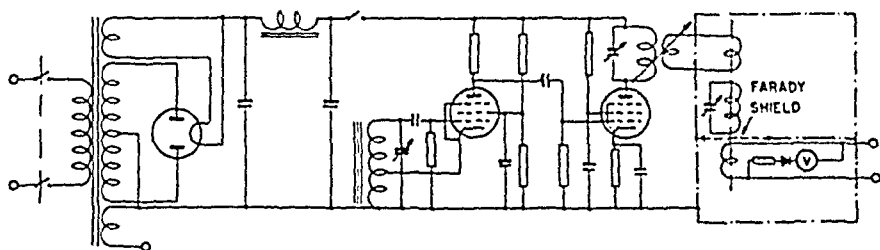


Fig. 2. Schematic drawing of diathermic heating apparatus.

eliminate capacitive currents, that might produce undesirable stimulating effects in the brain, the oscillator was provided with a filter constructed as shown in the schematic figure (fig. 2).

In control experiments the temperature changes in the brain tissue during heating were recorded with a thermocouple oriented with the electrode carrier of the HORSLEY-CLARKE apparatus to various points within the heated region. The relation between the voltage of the diathermic current and the increase of temperature with the thermocouple junction placed in a mid position between the electrode tips is seen in fig. 3. These experiments showed that the heat field between the tips was rather homogeneous. With a voltage of 14 volts the temperature increase at a point midway between the electrodes (6 mm apart) was after two minutes  $4.0^{\circ}\text{C}$ , while at a point 1.5 mm from one of the electrodes the temperature increase was  $4.7^{\circ}\text{C}$ . Moving the thermocouple in the sagittal or the vertical plane revealed a steep temperature gradient between the homogeneous field and its surroundings. For instance at a point in the midline 2.5 mm rostral to the frontal plane through the electrodes the temperature increased only  $1.8^{\circ}$  in two minutes when the diathermic voltage amounted to 14 volts. Thus the heating could be considered to be rather strictly confined to the region between the electrode tips. The possibility of an electrostatic pick-up in the thermocouple causing too high temperature readings cannot be excluded in the experiments with diathermic heating.

In most of the experiments the temperature of the heated hypothalamic structures was measured with the thermocouple junction situated midway between the tips of the electrodes. Body temperature was continuously observed with a mercury thermometer inserted high in the rectum. Efforts were made to keep body temperature at a con-

stant level by placing the cat on a heating pad, care being taken not to expose the limbs to direct heat.

Blood pressure was recorded in the left carotid artery by a mercury manometer.

Cutaneous blood flow was recorded, in the forelimb in the cephalic vein cannulated at the elbow, in the hindlimb in one of the saphenal

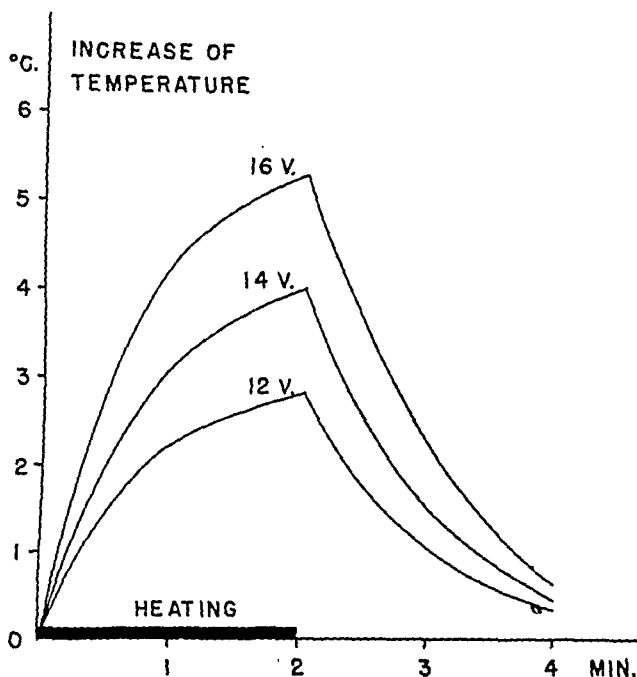


Fig. 3. Diagram illustrating the relation between diathermy voltage and temperature increase, measured with the thermocouple junction placed midway between the uninsulated tips of the electrodes. Electrodes placed in the anterior hypothalamus of a cat, with a distance between the electrodes of 6 mm. Stimulation time 2 minutes. The curves given are means of several experiments.

veins cannulated 2—3 cm distal to the knee. The venous outflow was directed to a photoelectric drop recorder connected with an "ordinate recorder".\* The venous outflow was returned to the animal by intravenous drop infusion. To prevent clotting heparin was given intravenously.

In a few experiments the blood flow was separately recorded in the abdominal inferior caval vein, and in a saphenal vein from a hindlimb of a cat. In these experiments the animals were eviscerated as described by FOLKOW and UVNÄS (1948). The caval outflow was measured with a Gaddum recorder, the saphenal flow with the drop recorder described above.

\* The ordinate recorder ("Ordinatenschreiber") is described in detail in another paper appearing in this volume (CLEMENTZ & RYBERG 1949). Since this instrument records the time intervals between drops, the height of the ordinates will be inversely proportional to the rate of blood flow.

Usually the stimulated brains were removed and prepared for microscopical examination.

## Results.

### *Experiments on cats.*

Observations were made on 49 cats. Pronounced cutaneous vasodilator responses were elicited on local heating of hypothalamic structures corresponding to the heat sensitive region described by MAGOUN et al. 1938. The lowest threshold to heat was found with the electrode tips placed 2 mm above the anterior border of the optic chiasma (in cats weighing about 3 kg this region corresponds to a point in the coordinate system of the Horsley-Clarke instrument 14—15 mm anterior to the interaural plane and 4—6 mm below the horizontal plane). In front of this region no vasodilator responses were obtained. On the other hand vasodilatation could be elicited from a region reaching 8 mm caudal to the anterior border of the chiasma, the threshold heat being markedly higher in the caudal parts. The vertical extension of the sensitive structures was observed to be about 2 mm. A further analysis of the localisation of the heat sensitive structures is postponed until histological examination of the material is complete.

Fig. 4. illustrates the cutaneous vasodilatation observed in a forelimb as the result of heating the hypothalamus with a diathermic current of various voltage. The silver electrodes were used in this experiment. As seen a stimulation with a voltage of 8 volts for one minute (corresponding to a temperature increase of not more than  $0.5^{\circ}\text{C}$ ) produces a slight vasodilatation that appears with a latency of about 15—20 seconds. The vasodilatation reaches its maximum gradually and disappears rapidly after the end of the stimulation. The vasodilator response increases with increasing diathermic voltage. At the same time the latency of the response decreases to a few seconds. When the vasodilatation reaches its maximum continued heating is able to maintain the increased flow at a constant level for a considerable period of time, in the experiment shown in fig. 4 for at least 3 minutes. Thus a vasodilatation of a certain magnitude can be produced either by a rapid temperature increase of short duration or by a slow temperature increase of long duration.



BLOOD PRESSURE

MM HG.

150-

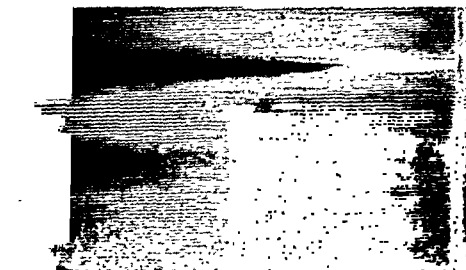
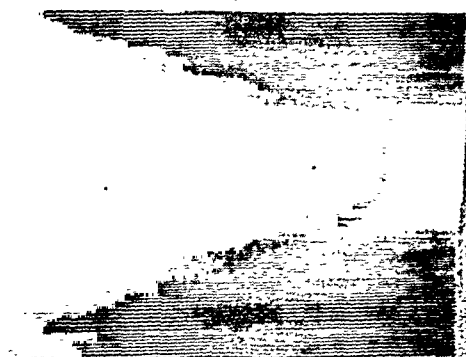
130-

110-



BLOOD FLOW

V. CEPHALICA DX

STIMULATION  
TIME 1 MIN.

1 5

4

3

2

1

It was frequently observed that the temperature in the hypothalamus was considerably below the rectal temperature. The difference usually amounted to about  $1^{\circ}\text{C}$ . A similar observation was made by SEROTA and GERARD (1938) who found the temperature of various parts of the brain in anaesthetized animals to be lower than the blood temperature. Since we found the observed difference of the temperature between the hypothalamus and the rectum surprisingly high the thermocouple readings were standardized against the rectal temperature. The readings were observed to be correct, however.

Attempts were made to correlate the magnitude of the vasodilator responses to the level of hypothalamic temperature reached during heating. When the electrodes were localized in the region most sensitive to heat an increase of temperature to about  $39^{\circ}\text{C}$  was sufficient to give an apparently maximal vasodilatation, provided that the rectal temperature was not below  $38^{\circ}\text{C}$ . If the body temperature was below  $38^{\circ}\text{C}$  or if a less sensitive region in the hypothalamus was reached warming to a higher temperature was necessary.

At point 5 in fig. 4 the silver electrodes were warmed by circulating hot water. The cutaneous outflow showed a tenfold increase, that gradually diminished when heating was discontinued. The observation justifies the conclusion that the stimulating effect of the diathermic current is due to the heat produced.

The vasodilatation induced by heating the anterior hypothalamus is confined to the cutaneous vessels. The exclusive localisation of the vasodilatation to the skin is already indicated by the fact that a pronounced increase of cutaneous blood flow occurs without any considerable or scarcely noticeable fall of blood pressure. In the experiment demonstrated in fig. 5 the blood flow was separately recorded in the caval vein, and in the saphenal veins of the hindlimbs. Local heating of the anterior hypothalamus elicits a marked increase of the saphenal outflow. Simultaneously a slight increase occurs in the caval flow. That this increase is due to the partial drainage of cutaneous veins into the caval vein is evident from the observations made later in the experiment.

Fig. 4. Cat 3.3 kg, chloralose + urethane, rectal temp.  $38.2^{\circ}\text{C}$ . Silver electrodes. Stimulation point at coordinates  $A = 15$ ,  $V = -4$  (anterior and vertical coordinates in the HORSLEY-CLARKE instrument). Stimulation nr 1—4 and 6: diathermic warming, nr 5: conduction warming.

Stimulation nr:	1	2	3	4	6
Diathermy voltage:	8	9	10	9	9
Temp. increase:	0.5	0.9	1.3	1.1	$1.1^{\circ}\text{C}$ .

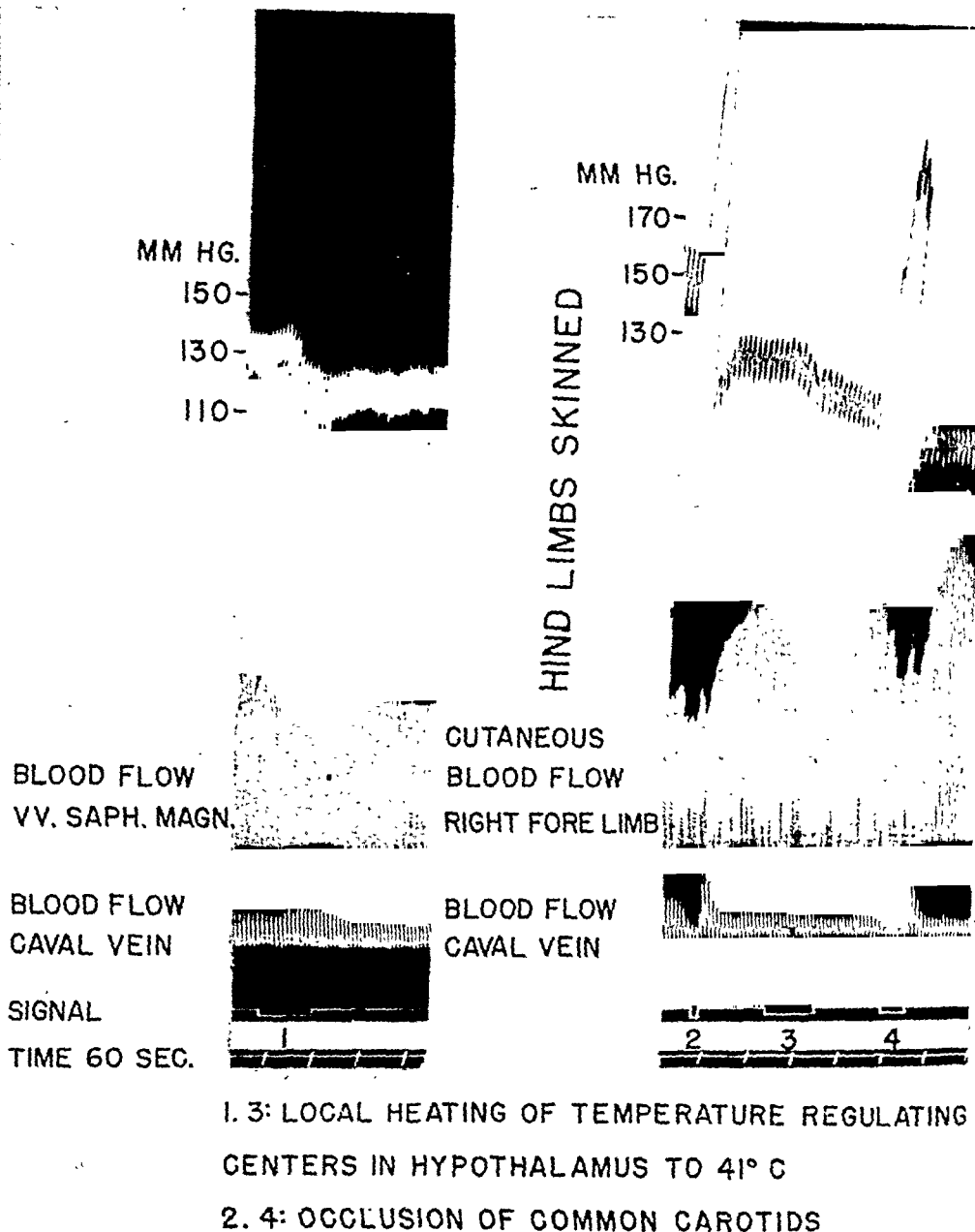


Fig. 5. Cat 3.2 kg, chloralose + urethane, rectal temp. 37.0° C. Nichrome electrodes, stimulation point at coordinates  $A = 15$ ,  $V = -6$ .

The hindlimbs were skinned and the cutaneous venous outflow now recorded from a forelimb. A repeated heating of the hypothalamus still induces a pronounced cutaneous vasodilatation. The

caval blood flow is now completely uninfluenced. That the efferent vasomotor pathways to the muscles are still intact is shown by the marked constrictor response that occurs on occlusion of the common carotids as well as by the vasodilatation consequent to the re-establishing of the carotid blood flow.

### *Experiments on dogs.*

In experiments on 6 littermate dogs weighing about 5 kg electrodes were placed in the hypothalamus as previously described in the experiments on cats. In the dogs an even more marked increase of cutaneous blood was observed on diathermic heating. The heat sensitive region was localized analogously with the same regions in cats.

No panting was observed to occur on heating the hypothalamus either in the cats or the dogs in spite of the fact that pronounced vasodilator responses occurred, indicating an activation of the central heat loss mechanism. The significance of this observation will be further discussed in a later paper from this laboratory.

### **Discussion.**

Our results are in accordance with the findings of MAGOUN et al. as far as the location of a heat sensitive region in the anterior part of the hypothalamus is concerned. The observations show that pronounced cutaneous vasodilator responses are induced by heating hypothalamic structures in cats and dogs. They further indicate that cutaneous vasodilatation constitutes an initial phenomenon when the central nervous heat loss mechanism is activated. The fact that the heat loss mechanism is activated by diathermic warming as well as by conduction warming in our view justifies the conclusion that increased temperature is the adequate stimulus of the neural mechanism concerned. The fact that a vasodilatation can be maintained at a constant level for a considerable time after the hypothalamic temperature has reached a plane above the threshold value indicates that the actual temperature and not the temperature gradient constitutes the adequate stimulus for the heat loss regulating neurones. This assumption is further strengthened by the observation that a vasodilator response of a certain magnitude is eventually reached

whether the temperature reaches its final level with a steep or slight gradient.

### Summary.

Local heating of the anterior part of the hypothalamus with diathermic or conducted heat induces cutaneous vasodilatation in the cat and dog.

Part of the expenses for this investigation was defrayed by a grant from the Medical Faculty of the University of Lund to one of us (G. S.).

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From the Department of Physiology, University of Lund.

## **Efferent Nervous Pathways Involved in Cutaneous Vasodilatation Induced by Activation of Hypothalamic Heat Loss Mechanisms.**

By

BJÖRN FOLKOW, GUNNAR STRÖM and BÖRJE UVNÄS.

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The cutaneous vessels of the cat and dog are amply supplied with constrictor fibres of sympathetic origin. Since there is no known sympathetic vasodilator innervation of the skin of these animals it is reasonable to assume that cutaneous vasodilatation is brought about by inhibition of the normally occurring tonic sympathetic discharge. However, in addition dorsal root fibres are supposed to play a part in centrally induced vasodilator reactions.

The idea that afferent fibres in the dorsal roots function as efferent pathways in a centrally directed vasodilator system was originally proposed by BAYLISS (1902, 1923). Most of the attempts to furnish additional evidence in support of this hypothesis have failed. Thus BACQ et al. (1934, 1939), THOMAS and BROOKS (1937), DOLE and MORISON (1940) and others were unable to find any support for BAYLISS' assumption that vasodilator fibres in dorsal roots are activated when depressor reflexes are elicited. A few investigators, among them FOFANOW and TSCHALUSSOW (1918), and TOURNADE and MALMÉJAC (1933) claim that vasodilator responses due to activation of depressor mechanisms remain after sympathectomy. This observation was taken to indicate the functional participation of antidromic vasodilator impulses in pressor and depressor reflexes.

BAYLISS claims that antidromic vasodilator impulses in the dorsal roots are conveyed principally to the splanchnic and cutaneous vessels. If this is so, these vasodilator impulses should be expected to participate in the cutaneous vasodilatation involved in the heat loss mechanisms. The present experiments were performed in order to investigate the rôle of dorsal root fibres as efferent vasodilator pathways in the heat regulating mechanism.

### Method.

Experiments were performed on cats and dogs under chloralose-urethane (30 + 300 mg/kg), chloralose (70 mg/kg), urethane (1 g/kg) or nembutal (30 mg/kg). The anaesthetics were given intravenously under an initial short ether anaesthesia. Most of the experiments were made under chloralose-urethane.

Cutaneous blood flow was recorded, in the forelimb in the cephalic vein cannulated at the elbow, in the hindlimb in one of the saphenal veins cannulated 2—3 cm below the knee. The venous outflow was directed to a photoelectrical drop recorder connected with an "ordinate recorder" (CLEMENTZ and RYBERG 1949), and then returned to the animal by intravenous drop infusion. To prevent clotting heparin was given intravenously.

In some of the experiments the hindlimbs were sympathectomized by removal of the abdominal sympathetic chains. These experiments were performed on eviscerated animals. The evisceration technique was described in detail by FOLKOW and UVNÄS (1948).

In a few experiments cross-circulation was arranged between two cats. The technique is previously described by FOLKOW and UVNÄS (1948, p. 372—73). *In principle*: Two cats were eviscerated. The recipient animal was divided in an upper and a lower part by complete ligation of skin and muscles at the height of the fifth lumbar vertebra. The vertebral column and the abdominal sympathetics were left intact. The cats were heparinized with 10 mg heparin per kg intravenously. The aorta and the inferior caval vein of both animals were ligated at the height of L<sub>5</sub>. The proximal end of the aorta of the donor was connected with the distal part of the aorta of the recipient. The venous outflow from the recipient's hindlimbs was directed to a Gaddum recorder through a cannula in the lower caval vein distally to the ligature. From the recorder the venous blood was returned to the proximal part of the ligated caval vein of the donor.

The cutaneous blood flow from the hindlimbs was recorded from one of the cannulated saphenal veins of each limb. The venous outflow from these veins was directed to a photoelectrical drop recorder connected with an ordinate recorder as described above and the venous blood then allowed to drain into the funnel of the Gaddum recorder. By this cross-circulation arrangement the isolated hind part of the recipient animal received blood practically solely from the donor,

whilst the nervous connexions with the upper part of the animal remained intact via the sympathetic chains and the spinal medulla. The cross-circulation arrangement had the advantage that vascular reactions in the upper part of the recipient animal were not able mechanically to influence the blood flow through the perfused hindlimbs. On the other hand, the central nervous vasomotor control of the vessels of the hindlimbs remained intact.

The blood pressures of both animals were measured by mercury manometers connected to the brachial arteries. Intraarterial injections to the hindlimbs were made into the cannulated stump of the inferior mesenteric artery.

Cutaneous vasodilatation was produced by local heating of the anterior part of the hypothalamus. Two electrodes were inserted into this region with the Horsley-Clarke technique and the neural structures between the electrodes heated with a diathermic current. As shown by FOLKOW *et al.* 1949 by this procedure an apparently maximal cutaneous vasodilatation can be induced. For details of the technique the paper by these authors can be referred to.

As reported by C. v. EULER (1947) it is possible selectively to activate thin afferent fibres in a mixed peripheral nerve, as for instance the sciatic, by thermal stimulation. In a few of the present experiments a thermode was placed round the sciatic nerve and afferent fibres activated by heating the thermode with circulating water to about 45° C.

## Results.

### *Experiments on cats.*

In experiments on 12 eviscerated cats the cutaneous blood flow was simultaneously recorded in a forelimb and a hindlimb. Local heating of the anterior hypothalamus produced a marked cutaneous vasodilatation in both limbs. Total sympathectomy of the hindlimbs by removal of the abdominal sympathetic chains from the height of the first lumbar vertebra downwards completely abolished the vasodilator response in the hindlimbs. As seen in fig. 1, due to loss of vasoconstrictor tone sympathectomy causes a considerable increase of cutaneous blood flow. The vasodilator action of acetylcholine diminishes, sometimes almost disappears (see for instance fig. 1). The disappearance of vasodilator responses to hypothalamic heat stimulation, therefore, might be explained as due to a decreased ability of postulated antidromic vasodilator impulses in the dorsal roots to produce further arteriolar dilatation. In order to re-establish arteriolar tone adrenaline was given in a continuous intravenous infusion. As illustrated in fig. 1 acetylcholine now induces a vasodilatation as pronounced



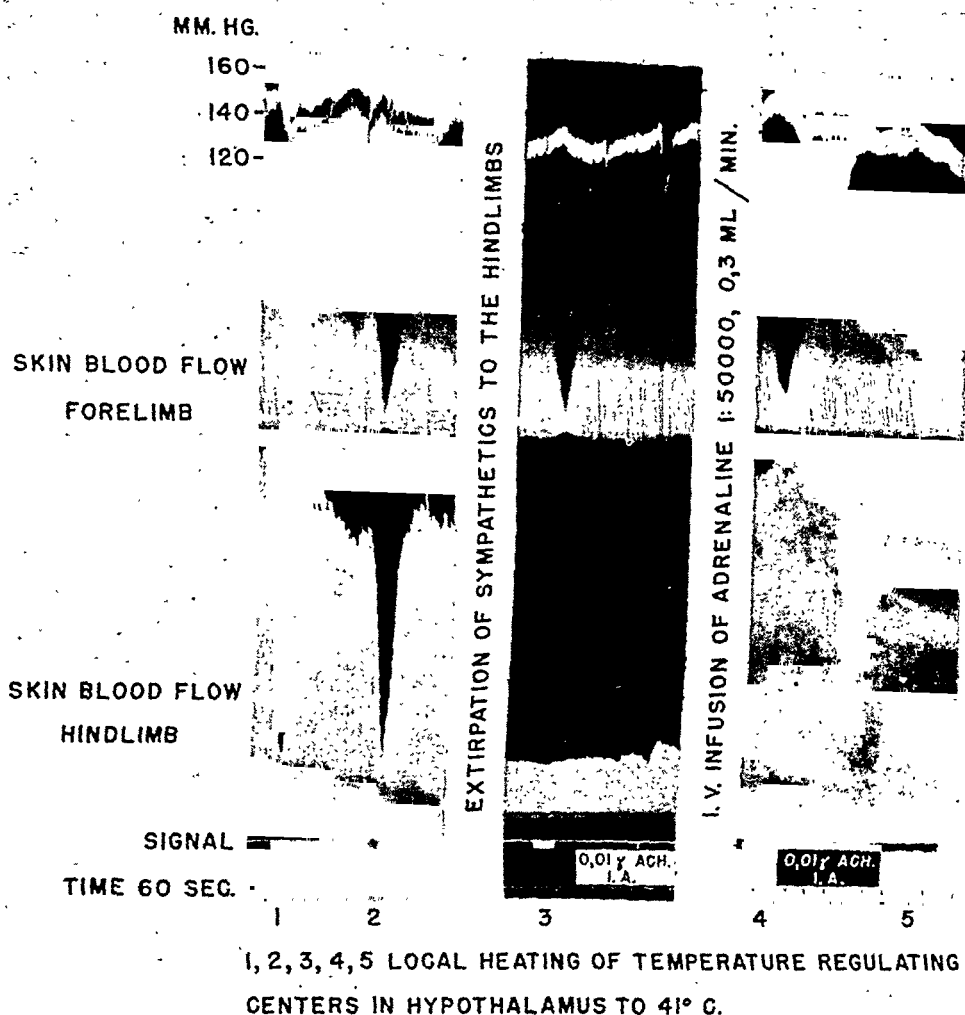


Fig. 1. Cat 2.5 kg. Chloralose-urethane, rectal temp. 38.5° C. Diathermy electrodes oriented to a point corresponding to the Horsley-Clarke ordinates A: 15, V: — 4, Voltage 11 V.

Cutaneous vasodilatation in fore- and hindlimb during diathermic heating of hypothalamus. Effect of sympathectomy of the hindlimbs.

as before sympathectomy. Still no vasodilator response to hypothalamic heating occurs.

The general cutaneous vasodilatation elicited by hypothalamic stimulation was accompanied by a slight fall of blood pressure, that might mask a minute vasodilator response in the sympathectomized hindlimbs. According to our experience such an explanation is rather improbable. However, in order to exclude even

BLOOD PRESSURE

MM HG.

DONOR.

160-

140-

RECIPIENT

170-

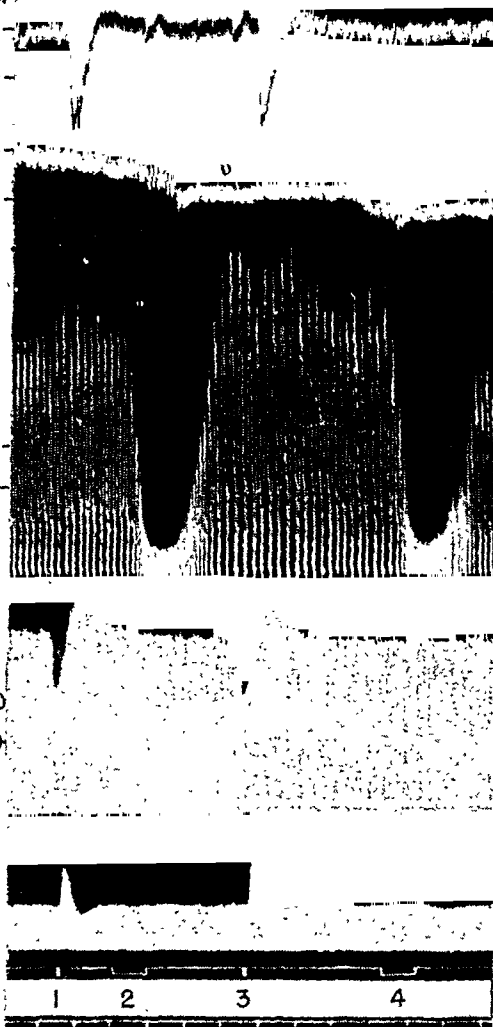
150-

CUTANEOUS BLOOD-  
FLOW RIGHT FORE-  
LIMB

CUTANEOUS BLOOD  
FLOW SYMPATHECTO-  
MIZED HINDLIMBS

BLOOD FLOW  
CAVAL VEIN  
SIGNAL

TIME 60 SECONDS



1. 3: INJECTION OF 0.1 $\gamma$  ACETYLCHOLINE INTO  
INFERIOR MESENTERIC ARTERY
2. 4: LOCAL HEATING OF TEMPERATURE  
REGULATING CENTERS IN HYPOTHALAMUS  
TO 41° C

Fig. 2. Cross-circulation. Chloralose-urethane. Donor cat 5.2 kg, recipient cat 4.2 kg. Diathermy electrodes oriented to a point corresponding to the Horsley-Clarke ordinates A: 15, V: — 4. Voltage 15 V.

Hindlimbs of the recipients sympathectomized.  
Cutaneous vasodilatation in forelimb during diathermic heating of hypothalamus. Note the absence of vasodilator responses in the hindlimbs.

this remote possibility a few observations were made on cross-circulated animals.

Fig. 2 shows a cross-circulation experiment. Cutaneous blood flow is simultaneously registered from the normally innervated

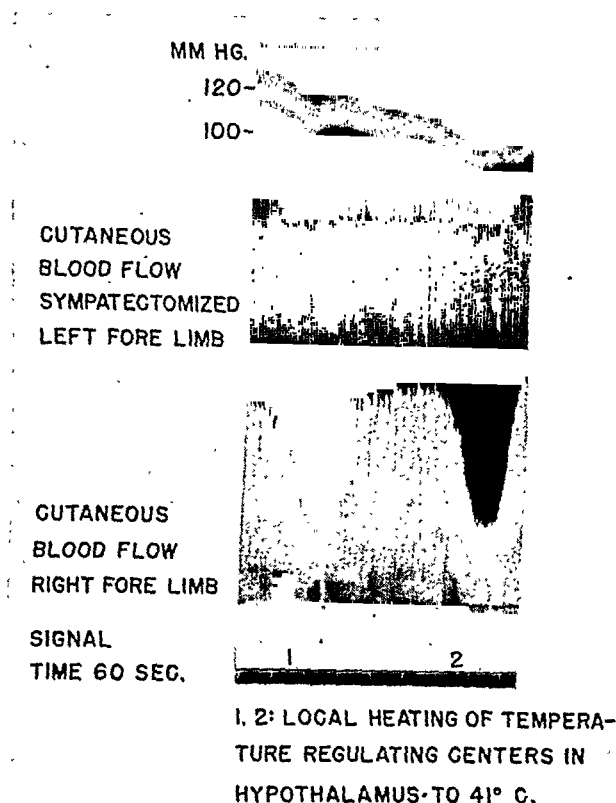


Fig. 3. Cat 3.1 kg. Chloralose-urethane. Rectal temp. 36.0° C. Diathermy electrodes oriented to a point corresponding to the Horsley-Clarke ordinates A: 16, V: — 4, Voltage 16 V.

Left forelimb sympathectomized by removal of stellate ganglion 10 days prior to experiment.

Note the absence of vasodilator response in sympathectomized forelimb during diathermic heating of hypothalamus.

right forelimb and the sympathectomized hindlimbs. In addition the caval outflow is recorded.

Hypothalamic heating elicits a pronounced increase of cutaneous blood flow in the forelimb. No vasodilator response occurs in the hindlimbs. On the other hand acetylcholine given intraarterially produces a marked vasodilatation of the cutaneous as well as of the muscular vessels.

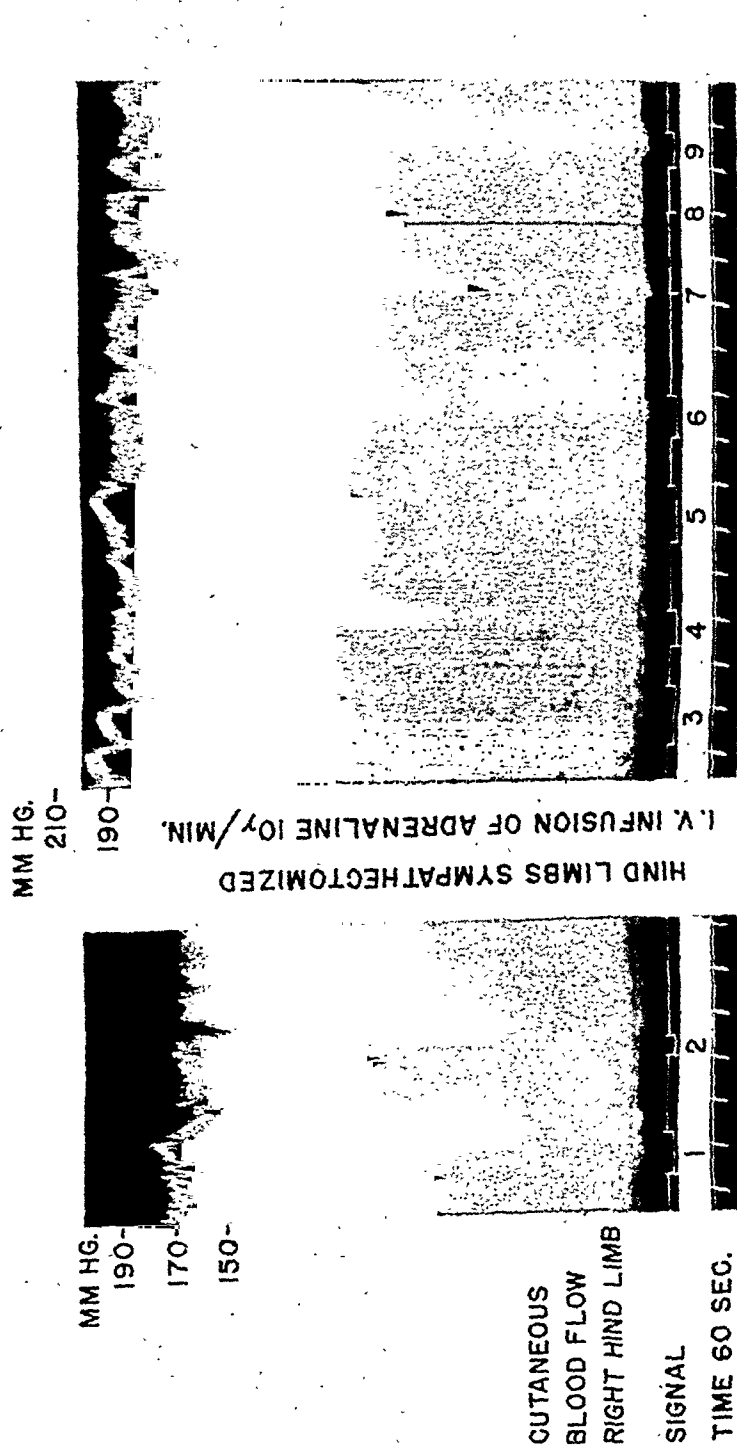
In cats and dogs sympathectomized arterioles regain their tone in a few days. Sympathectomy of the left forelimb was performed on 5 cats by removal of the left stellate ganglion. The ganglion was reached by an extrapleural approach in the interscapular region.

The animals were used for experiment 10—14 days after sympathectomy. The cutaneous blood flow was simultaneously recorded in the left and the right forelimb. As seen in fig. 3 heating the hypothalamus elicits a pronounced increase of cutaneous blood flow in the right limb. The blood flow in the sympathectomized left limb remains completely unchanged. Identical observations were made on all 5 animals.

*Antidromic vasodilatation in sympathectomized skin.*

It might be argued that the absence of cutaneous vasodilator responses to hypothalamic stimulation in sympathectomized limbs was due to the fact that the afferent nerve fibres mediating the postulated antidromic dilatator impulses were put out of function by the experimental procedures. In our opinion the validity of this argument is considerably reduced by the observation that at the end of our experiments afferent stimulation of a spinal nerve still was able to elicit reflex vegetative and somatic reactions. To further demonstrate the functional integrity of the nervous pathways assumed to transmit antidromic vasodilator impulses, the following experiments were made on a few cats (fig. 4). Cutaneous blood flow was measured in the v. saphena parva of the right hindlimb. Hypothalamic heating produced a marked cutaneous vasodilatation. By means of a thermode the right sciatic nerve was locally heated to 45° C. This procedure selectively activates the afferent fibres of a mixed nerve (C. v. EULER 1947) and as seen in fig. 4 the antidromically conducted discharge elicited a cutaneous vasodilatation.

The hindlimbs were now sympathectomized. The consequent loss of constrictor tone in the denervated vessels was compensated by a continuous intravenous infusion of adrenaline 10  $\gamma$ /min. The vasodilator response to hypothalamic heating completely disappeared after sympathectomy. Thermal heating of the right sciatic nerve, either cut proximally to the point of stimulation or with its afferent connexions intact, still produced an undiminished vasodilatation. Mechanical stimulation of the sciatic by pinching the nerve with a pair of forceps, a procedure that ac-



1. 3. 5: LOCAL HEATING OF TEMPERATURE REGULATING CENTERS IN HYPOTHALAMUS

TO  $41^{\circ}\text{C.}$

2. 4. 6. 9: THERMAL STIMULATION OF RIGHT SCIATIC NERVE

7. MECHANICAL STIMULATION OF RIGHT SCIATIC NERVE

8: SECTION OF RIGHT SCIATIC NERVE PROXIMALLY TO POINT OF THERMAL STIMULATION

cording to BAYLISS and others activates the dilator fibres of the dorsal roots, caused a marked vasodilatation of the skin vessels.

*Experiments on dogs.*

The effect of sympathectomy on cutaneous vasodilator responses to hypothalamic heating was studied in experiments on 4 littermate dogs. The technique was the same as previously described for the cats. As shown in fig. 5 sympathectomy of the hindlimbs completely abolishes the vasodilator responses in these limbs. The vasodilator response of the cutaneous vessels to acetylcholine remains after sympathectomy indicating the ability of the vessels to respond to dilator stimuli. C in the figure indicates that the slight blood pressure fall accompanying the general cutaneous vasodilatation under hypothalamic heating was compensated by a slow intravenous infusion of blood. Preventing the fall of blood pressure did not reveal any masked cutaneous vasodilatation.

That hypothalamic heating is still able to activate the heat loss mechanisms is evident from the record at the extreme right of the figure, where the cutaneous flow in a forelimb is shown.

### Discussion.

The experiments have shown that cutaneous vasodilatation induced in cats and dogs by local diathermic heating of the anterior hypothalamus is completely abolished by sympathectomy. The vasodilator responses regularly disappear whether the sympathectomy is performed during the course of the experiment and the vascular tone is re-established by intravenous infusion of adrenaline or if the sympathectomy is performed 10 days prior to the experiment thus allowing the vessels to regain their tone spontaneously.

Our experiments were made in order to investigate the possible rôle of the postulated antidromic vasodilator impulses in dorsal roots in producing cutaneous vasodilatation. Since the absence of vasodilator responses after sympathectomy might be ascribed to

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Fig. 4. Cat 2.8 kg. Chloralose-urethane. Rectal temp. 37.5° C. Diathermy electrodes oriented to a point corresponding to the Horsley-Clarke ordinates A: 14, V: — 4, Voltage 14 V. (at 1), 16 V. (at 3 and 5).

Effect of sympathectomy on cutaneous vasodilator responses in right hindlimb to diathermic heating of hypothalamus, and to thermal and mechanical stimulation of right sciatic nerve.

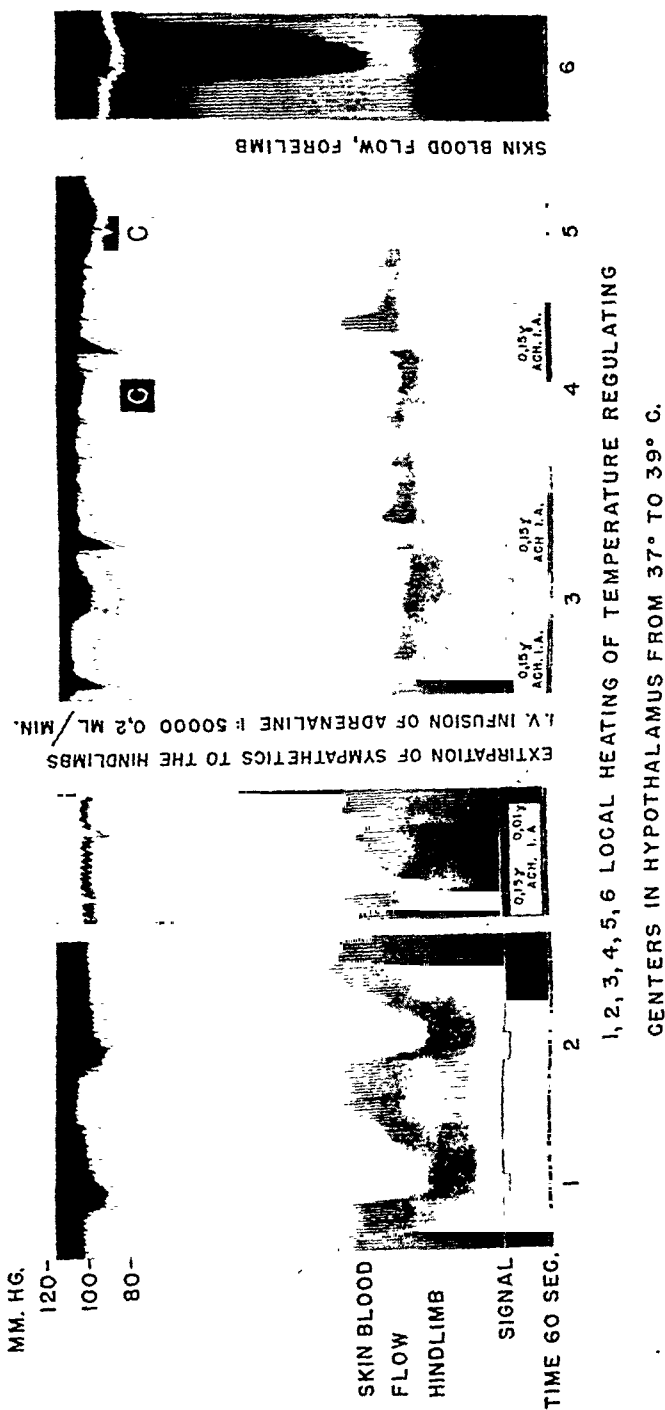


Fig. 5. Dog 4.5 kg. Chloralose-urethane. Rectal temp. 38.0° C. Diathermy electrodes oriented to a point corresponding to the Horsley-Clarke ordinates A: 21, V: —6, Voltage 11 V.

The effect of sympathectomy on cutaneous vasodilatation induced by diathermic heating of hypothalamus. Acetylcholine was injected into the inferior mesenteric artery.

a blocking of the afferent pathways involved in antidromic vasodilatation the functional integrity of these pathways was demonstrated by selective activation of the thin afferent fibres in a mixed spinal nerve. This procedure produced an antidromic discharge of vasodilator impulses and simultaneously a reflex pattern of somatic and vegetative reactions. In our view this observation excludes dorsal root fibres as nervous pathways in the production of cutaneous vasodilatation induced by activation of the central nervous heat loss mechanism. Since it is generally assumed that in cats and dogs cutaneous vessels are devoid of any sympathetic vasodilator innervation, it appears as if centrally induced cutaneous vasodilatation is effected solely by reduction of sympathetic constrictor tone. LEWIS and PICKERING (1931), and later FATHERREE and ALLEN (1936) obtained from experiments on patients with RAYNAUD's disease some evidence that, according to these authors, indicated the existence of sympathetic vasodilator fibres to the skin of fingers and toes. Other investigators have failed to confirm these observations (WARREN et al. (1942), SARNOFF and SIMEONE (1941) and others). GRANT and HOLLING claim that sympathetic fibres run to the skin of the human forearm. On heating human beings they observed a cutaneous vasodilatation in the forearm that was considered to be too extensive to be explained merely as the consequence of the disappearance of vasoconstrictor tone. With the exception of these few observations all attempts to show the existence of sympathetic vasodilator fibres to the human skin vessels have failed. As long as there is no proof that such vasodilator fibres exist in the human it seems reasonable to assume that central nervous influences on the cutaneous blood flow is mediated solely by variation of sympathetic vasoconstrictor tone. In our opinion the speculations about the possible rôle of centrally induced antidromic vasodilator impulses transmitted in dorsal roots are not supported by any conclusive experimental evidence. The participation of such impulses in depressor reflexes will be discussed in a later paper.

### Summary.

In the cat and dog cutaneous vasodilator responses induced by activation of hypothalamic heat loss mechanisms are completely abolished by sympathectomy.



No evidence was obtained to indicate the participation of dorsal root dilator fibres in centrally induced cutaneous vasodilatation.

Part of the expenses for this investigation was defrayed by a grant from the Medical Faculty of the University of Lund to one of us (B. F.).

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From the Department of Physiology, University of Lund.

## An Ordinate Recorder for Measuring Drop Flow.

By

BENGT CLEMENTZ and CARL-EGON RYBERG.

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The ordinate recorder to be described below is the result of a rather long period of experimenting. In its final form it has functioned satisfactorily during several months of daily use. In a preliminary form this recorder was used by FOLKOW et al. (1949) in measuring blood drop flow. In construction it differs considerably from earlier types of ordinate recorders (FLEISCH 1930).

The frequency range of the recorder is 5—250 impulses per minute, and the lever's rate of rising can be shifted in 6 gear steps. As the recorder has been used mostly for drop flow recording, its electronic equipment has been constructed specially for this purpose. The drops are made to break a light beam lighting a phototube, thereby inducing an impulse which is amplified and made to energize a relay managing the mechanical recording system.

A phototube counter has a certain advantage over other types of electrical drop counters. Electrolysis of the dropping fluid with subsequent coating of the contact areas is avoided. The conducting properties of the fluid are unimportant. Finally, electrical insulation between electronic equipment (line) and dropping fluid (experimental animal) is complete.

As illustrated by fig. 1 the instrument operates as follows. The lever (1) is attached to the perforated steel band (9), moved over the sprocket wheel (7) and the blank wheel (12) by the rubber coated shaft (5) in the direction of the arrow. The shaft is driven

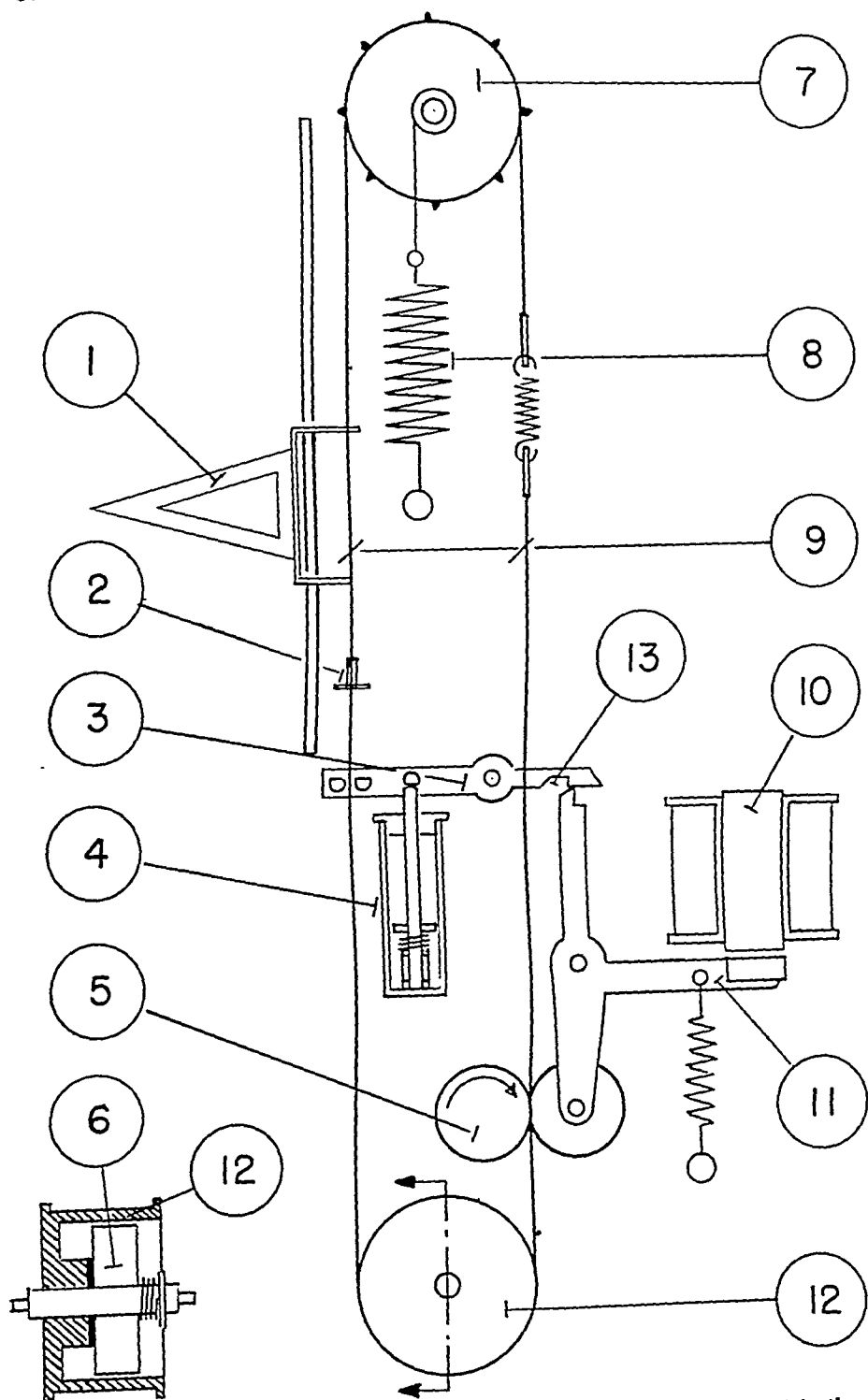


Fig. 1. Principal drawing of the ordinate recorder, with the armature (11) in the unlocked position. For explanation see text.

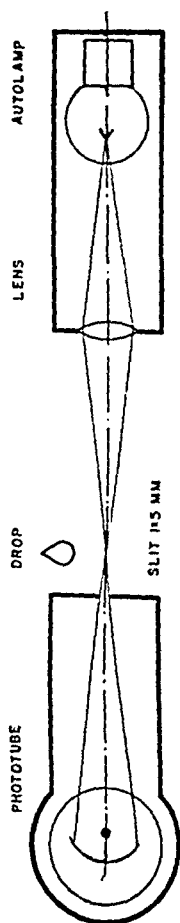


Fig. 2. Principal drawing of phototube counter.

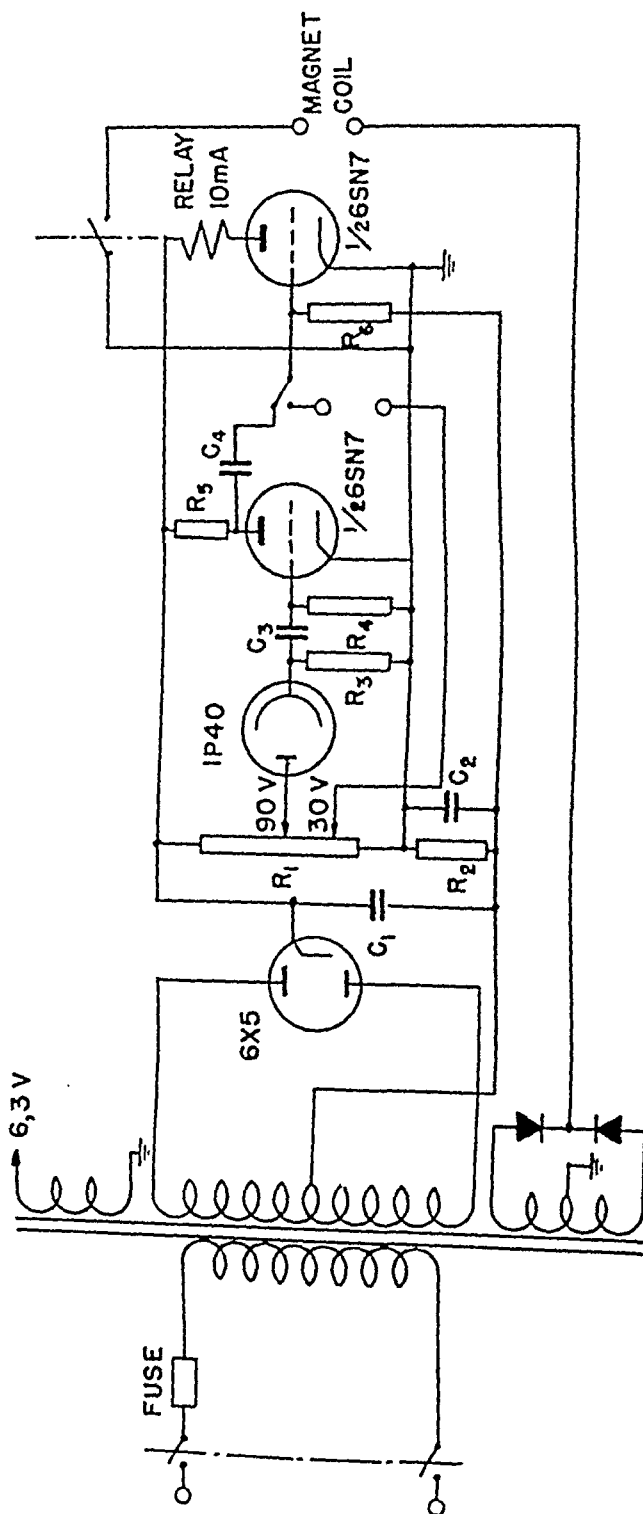


Fig. 3. Electronic equipment of the ordinate recorder. R<sub>1</sub>: 10 K $\Omega$ , R<sub>2</sub>: 25 $\Omega$ , R<sub>3</sub>: 5 M $\Omega$ , R<sub>4</sub>: 500 K $\Omega$ , R<sub>5</sub>: 100 K $\Omega$ , R<sub>6</sub>: 500 K $\Omega$ , C<sub>1</sub>: 40  $\mu$ F 450 V, C<sub>2</sub>: 50  $\mu$ F 50 V, C<sub>3</sub>: 0.1  $\mu$ F paper, C<sub>4</sub>: 0.5  $\mu$ F paper. Transformer: 2  $\times$  250 V 40 mA, 6.3 V 3 A, 2  $\times$  8 V 0.5 A.

by a centrifuge-regulated gramophone motor with a 6 step gear shift.

When an impulse from the phototube magnetizes the coil (10), the armature (11) is drawn upwards, releasing the steel band from

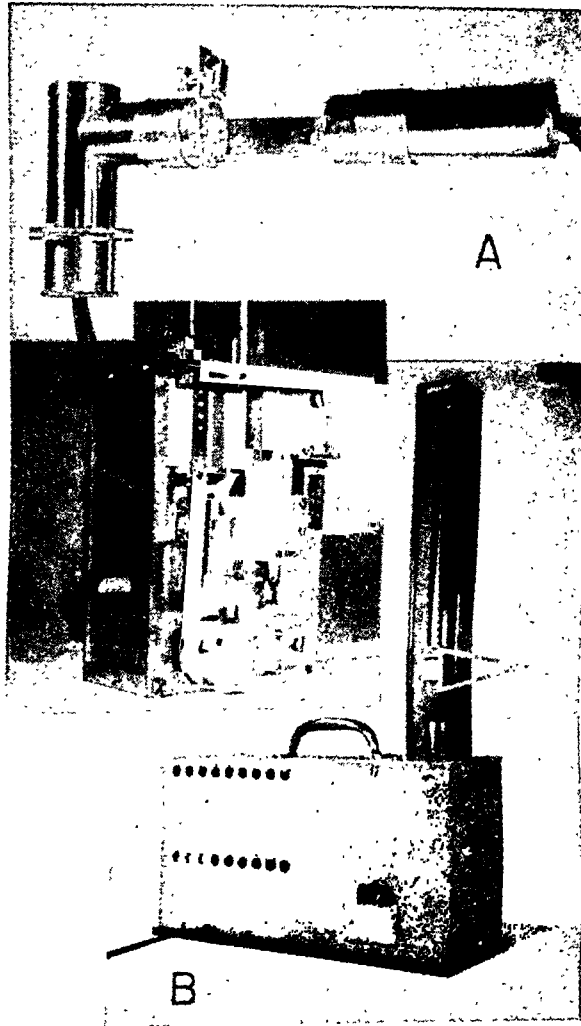


Fig. 4. A. Exterior view of phototube counter. B. Exterior view of ordinate recorder. C. Detail view of ordinate recorder, referable to fig. 1.

the friction against the rubber shaft. The steel band and the lever are now pulled back to zero position by the zero spring (8). At the same time the lock (13) is put into action, keeping the armature in the upward position. When the lever reaches zero, a release nut (2) on the steel band hits two pins placed on the lock arm (3),

the armature is released and the band is again moved by the rubber shaft, giving the lever a constant speed upwards from zero position. This device ensures the release of the steel band during the time when the lever is pulled down to zero, and makes the instrument independent of the duration of the incoming impulse. In order to procure smooth recording, the lock arm is damped by an oil damper (4). For the same reason an inertia wheel (6) is placed in the blank wheel.

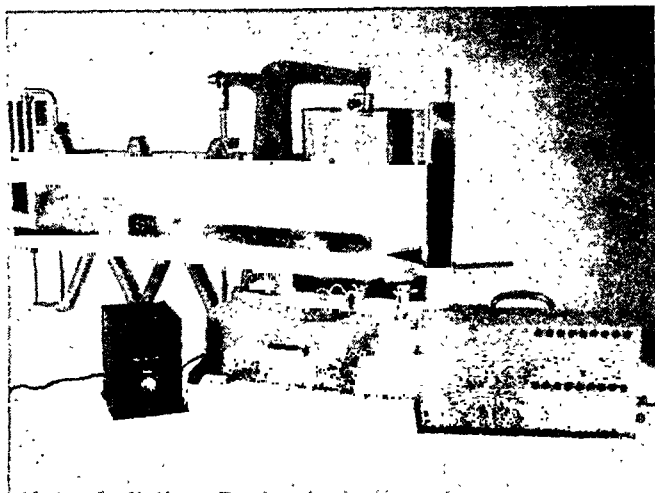


Fig. 5. View of the ordinate recorder recording on a smoked drum.

If the constant upward movement of the lever is not interrupted by an incoming impulse, the lever stops at a top position at a height of 12 cm above zero. This is accomplished by an extensive perforation in the steel band situated in its posterior part. When this perforation reaches the rubber shaft, the shaft loses contact with the band, and the band and the lever reach a standstill.

As mentioned above, the electronic equipment of the instrument is specially constructed for drop flow recording. It consists of a light-operated relay, built to allow its use as a common tube relay after switching. Electrical and mechanical construction is illustrated by figures 2—4.

The above described ordinate recorder has a small disadvantage, however. To allow a smooth recording and an almost noiseless operation, the zero spring has been given only a moderate tension. A rather slow return of the lever to zero is the result. Thus the height of the ordinates will not be directly proportional

to the time intervals between the drops. With the recorder geared at lowest speed the maximal error is 6 %, at highest speed the maximal error is 15 %. This error is usually without importance, as the recording generally aims at showing relative changes in drop flow.

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From the Department of Physiology, University of Lund.

## Effects of Antihistamine Compounds on the Adrenaline Liberation from the Suprarenals.

By

NILS EMMELIN and ANDERS MUREN.

Received 16 February 1949.

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In the last years a great number of compounds have been synthesized which strongly and specifically antagonize the action of histamine on blood vessels and smooth muscles. The analysis of the properties of these so-called antihistamine drugs has revealed the puzzling fact that all these substances, which are not all closely chemically related, are unable to prevent the stimulating action of histamine on gastric secretion. It has therefore been suggested "that histamine acts on secretory cells through some mechanism which differs from that obtaining in endothelial and smooth muscle cells; or, antihistamine drugs do not reach or have no affinity for the elements in the secretory cell which react to histamine" (LOEW 1947). Obviously it must be of interest to examine the effect of the antihistamine drugs on other secretory processes stimulated by histamine; but unfortunately the action of histamine on e. g. salivation, lacrymation, or secretion of pancreatic juice is not very pronounced and highly variable. It is well known, however, that histamine in small doses causes an output of adrenaline from the suprarenals (for references see SZCZYGIELSKI 1932) and it has frequently been suggested by DALE and his coworkers that this is a phenomenon of genuine physiological importance. The present paper deals with the action of antihistamine compounds on the secretion of adrenaline from the suprarenal medulla elicited by histamine and some other drugs.



## Methods.

27 cats in chloralose anaesthesia were used for these experiments. The method of FELDBERG and MINZ (1931) for intraarterial injections close to the suprarenals was adopted. The cats were eviscerated, the aorta and the vena cava were tied below the suprarenals, the renal vessels were ligatured, and a cannula was inserted in the coeliac artery. The vagi were cut in the neck. In many experiments the coeliac ganglion was removed, care being taken not to damage the vessels of the suprarenals. The blood pressure in a carotid artery was recorded. Contractions of the nictitating membrane, denervated by aseptic removal of the superior cervical ganglion 2—3 weeks previously were recorded in some cases. The following antihistamine compounds were examined: thymoxyethyldiethylamine HCl (929 F), N-phenyl-N-benzyl-N<sup>1</sup>, N<sup>1</sup>-dimethylethylenediamine HCl (antergan), 2-N-phenyl-N-benzylaminomethylimidazoline (antistine),  $\beta$ -dimethylaminoethyl benzhydrol ether HCl (benadryl), N- $\alpha$ -pyridyl-N-p-methoxybenzyl-N<sup>1</sup>, N<sup>1</sup>-dimethylethylenediamine H<sub>3</sub>PO<sub>4</sub> (neo-antergan), and N- $\alpha$ -pyridyl-N-benzyl-N<sup>1</sup>, N<sup>1</sup>-dimethylethylenediamine HCl (pyribenzamine).

## Results.

### 1. Antagonism between Histamine and Antihistamine Drugs.

Histamine was given in doses of 0.1—2  $\gamma$ , usually 0.2—0.4  $\gamma$ . It was injected intraarterially into the coelic artery. The response to these doses of histamine varied, as has been described by earlier investigators. Sometimes there was a pure rise in blood pressure starting about 15 seconds after the injection and accompanied by increased heart rate. Often the rise was preceded by a more or less pronounced fall in blood pressure; this was the case particularly in those experiments in which the initial blood pressure was high. Usually the rise in blood pressure caused by histamine and due to liberated adrenaline was pronounced but in some experiments it was very small even if the sensitivity to injected adrenaline was found to be high; apart from this there were some instances in which the sole or main effect of injected adrenaline was to cause a fall in blood pressure and on these oc-

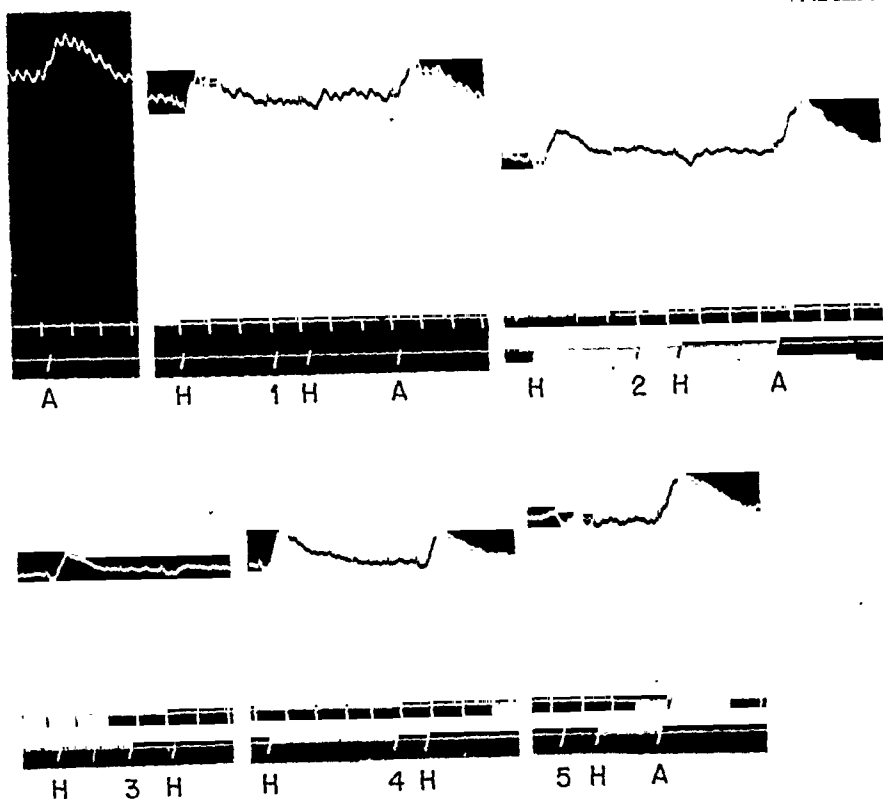


Fig. 1. Cat 2.4 kg. Injections into the coeliac artery of 3  $\gamma$  adrenaline at A, of 0.6  $\gamma$  histamine at H; of 0.01 mg pyribenzamine at 1; of 0.01 mg neoantergan at 2; of 0.01 mg benadryl at 3; of 0.01 mg antistine at 4 and of 0.1 mg antistine at 5. Between the second and the third section of the tracing 0.01 mg pyribenzamine was given intravenously 1 minute before section three starts. Interval between the different sections about ten minutes. Time in minutes.

casions histamine had naturally mainly a depressor effect. Obviously the blood pressure is not an ideal test for adrenaline, liberated by histamine, and we have therefore in some experiments in addition recorded the contractions of a nictitating membrane sensitized to adrenaline by previous denervation. In all experiments the sensitivity of the blood pressure to injected adrenaline was tested; this was necessary not only for reasons referred to above but also because antihistamine drugs are known to be able to affect the adrenaline sensitivity of the blood vessels. The injections of adrenaline were always made into the coeliac artery.

Usually the antihistamine drugs were also injected into the coeliac artery but sometimes for comparison intravenously. They were found strongly to antagonize the adrenaline releasing effect

of histamine. From fig. 1 it can be seen that doses as small as 10  $\gamma$  of benadryl, neoantergan and pyribenzamine given intraarterially abolish the adrenaline liberating effect of 0.6  $\gamma$  of histamine. This dose of antistine is without effect; the dose has to be increased to 0.1—0.5 mg in order to have an antagonistic action. Antergan acts in doses of the same order as antistine, whereas 929 F has to be given in a dose of about 0.5 mg to abolish the adrenaline liberating effect of histamine. When these small doses of antihistamine drugs are used the antagonistic effect is of short duration, lasting for 5—10 minutes only. The sensitivity to adrenaline is not affected. No antagonism can be demonstrated if the drugs are given intravenously in these small doses. In most experiments bigger doses, usually 0.5—2 mg, were given intraarterially and in these cases the antagonism lasted for several hours, usually throughout the experiment. Antistine seemed to be an exception; the effect even of 3 mg lasted only for about half an hour.

Apart from the direct effect of histamine on the cells of the suprarenal medulla there may sometimes be an indirect effect, as histamine by lowering the blood pressure may reflexly cause an output of adrenaline. This effect was discovered already by ELLIOTT in 1912. It is reasonable to expect the antihistamine drugs to antagonize this indirect effect of histamine if they are given in a dose sufficiently high to prevent a fall in blood pressure after histamine injections. But our experiments show that the drugs also antagonize the direct effect of histamine on the medullary cells. This is obvious from the following observations:

1. The antihistamine drugs prevent the adrenaline liberation by histamine in those experiments in which histamine does not elicit a fall in blood pressure.

2. Small doses of the drugs are more active in antagonizing the adrenaline release if given in the coeliac artery than intravenously. In experiments in which histamine initially lowers the blood pressure antihistamine drugs given in small doses intraarterially, close to the adrenals, are able to annul the pressor but not the depressor effect of histamine. These observations indicate that the action of the drugs in these instances is localized to the suprarenals and not to those receptors in the peripheral circulation on which histamine exerts its action in the small vessels. In experiments to be described below it will be shown that the antihistamine drugs do not antagonize the adrenaline liberating effect of acetylcholine; and the fact that small doses of the drugs given

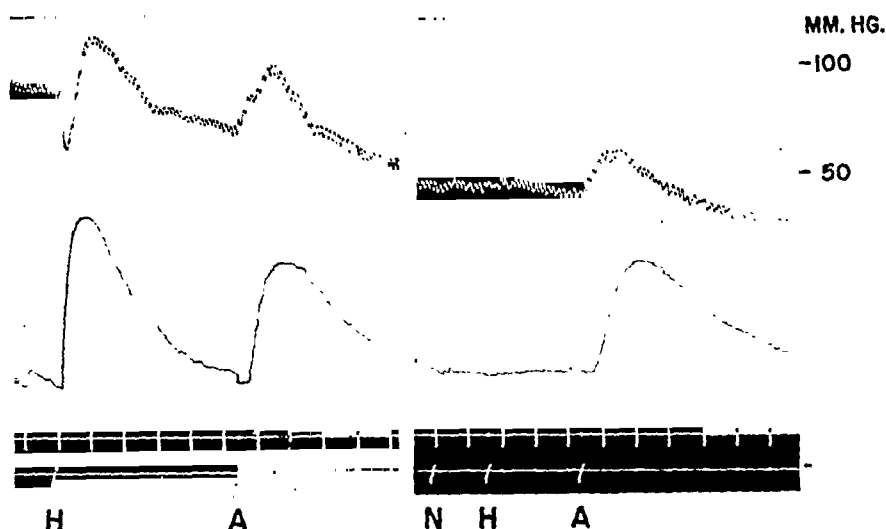


Fig. 2. Record of blood pressure (upper tracing) and contractions of denervated nictitating membrane (lower tracing) of a 2.2 kg cat with denervated suprarenals. Injections into the coeliac artery of 0.4  $\gamma$  histamine at H, 2  $\gamma$  adrenaline at A, 0.2 mg neoantergan at N. Time in minutes.

intraarterially are nevertheless able completely to abolish the pressor effect of histamine without necessarily preventing its depressor action may indicate that with our small doses of histamine given into the coeliac artery the adrenaline liberation is mainly or wholly due to a direct effect of the histamine on the suprarenal medulla.

3. In ten experiments the suprarenals were denervated by removing the coeliac ganglion. Even in these experiments in which a reflex action of histamine on the adrenals was thus excluded, the antihistamine drugs prevented liberation of adrenaline by histamine. This is demonstrated in fig. 2 which shows that 0.2 mg of neoantergan injected into the coeliac artery render 0.4  $\gamma$  of histamine injected close to the denervated glands ineffective on the blood pressure and the sensitized nictitating membrane without interfering with the response to adrenaline.

## 2. Specificity of the Antagonism.

Several substances are known to liberate adrenaline from the suprarenals, *e. g.* acetylcholine (DALE 1914, FELDBERG and MINZ 1931), lysolecithin (FELDBERG 1940) and potassium chloride. Also some bile salts cause this effect if a relatively big dose is given

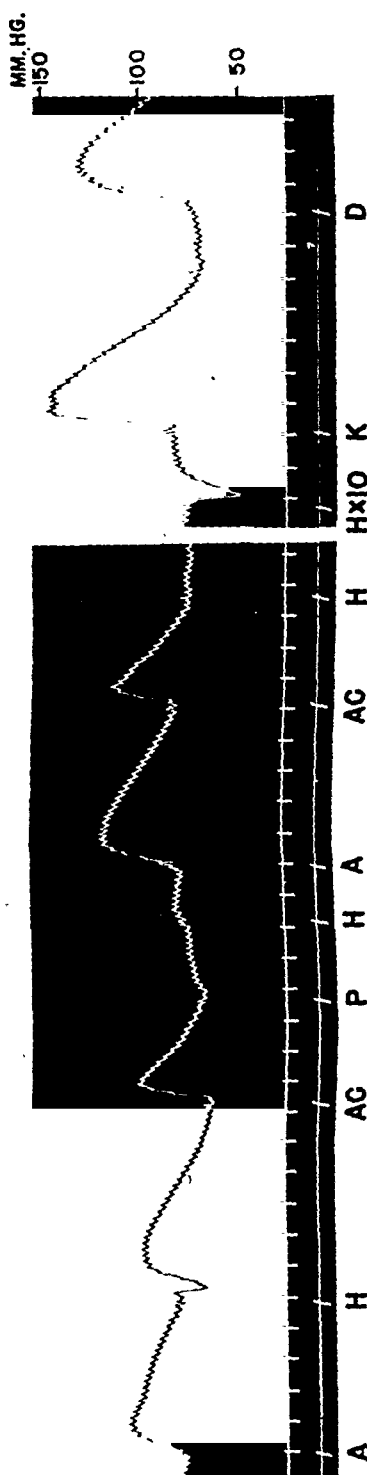


Fig. 3. Blood pressure record from 2.7 kg cat with denervated suprarenals. 1 mg atropine injected intravenously at the beginning of the experiment. Injections into the coeliac artery of 3  $\gamma$  adrenaline at A, 0.4  $\gamma$  histamine at H, of 4  $\gamma$  histamine at H  $\times$  10, 4  $\gamma$  acetylcholine at AC, 0.2 mg pyribenzamine at P, 3 mg KCl at K and 0.5 mg sodium desoxycholate at D.

(EMMELIN and MUREN 1949). We have examined whether the antihistamine drugs affect the release of adrenaline caused by acetylcholine (1—10  $\gamma$ ), KCl (0.5—3 mg) and bile salts injected intraarterially. The experiments show that the antihistamine compounds in doses which strongly antagonize the action of histamine on the suprarenals only slightly or not at all affect the action of acetylcholine, KCl and bile salts. In the experiment of fig. 3 0.4  $\gamma$  histamine and 4  $\gamma$  acetylcholine caused an output of adrenaline from the suprarenals. After 0.2 mg of pyribenzamine 0.4  $\gamma$  of histamine had scarcely any effect and ten times the dose had a pure depressor action; acetylcholine was still active. 3 mg of KCl and 0.5 mg of sodium desoxycholate both liberated adrenaline.

### 3. Liberation of Adrenaline by Antihistamine Compounds.

In the course of the experiments it was observed that 3 mg of neoantergan injected into the coeliac artery caused a rise in blood pressure which seemed to be due to liberated adrenaline. This finding seemed to suggest that the antihistamine compounds not only compete with histamine for the same receptors in the medullary cells, thus preventing histamine from being attached to the cells and exert its action; but when fixed to the receptors these drugs have the same action as histamine on the medullary cells. We therefore thought it worth while to examine this action of the drugs on the adrenals. It was found, however, that this similarity in action between histamine and antihistamine drugs is only superficial. Not all of the compounds studied have this action. The most potent adrenaline liberators seem to be pyribenzamine and benadryl. The smallest active doses are of the order of 0.1—0.5 mg injected into the coeliac artery. 1—3 mg of these drugs cause a great output of adrenaline. Antergan and neoantergan act in doses of the order of 2—5 mg. With 929 F and antistine doses as big as 5—10 mg have been tried. These big doses may cause a fall of the blood pressure and we have therefore also used the denervated nictitating membrane as a test. Unfortunately big doses of these drugs diminish the sensitivity of our test objects to adrenaline which makes the interpretation of the results more difficult, but no experiments indicate that 929 F or antistine are able to liberate adrenaline. Fig. 4 illustrates the action on the blood pressure of the different antihistamine compounds injected into the coeliac artery in big doses. Fig. 5 shows

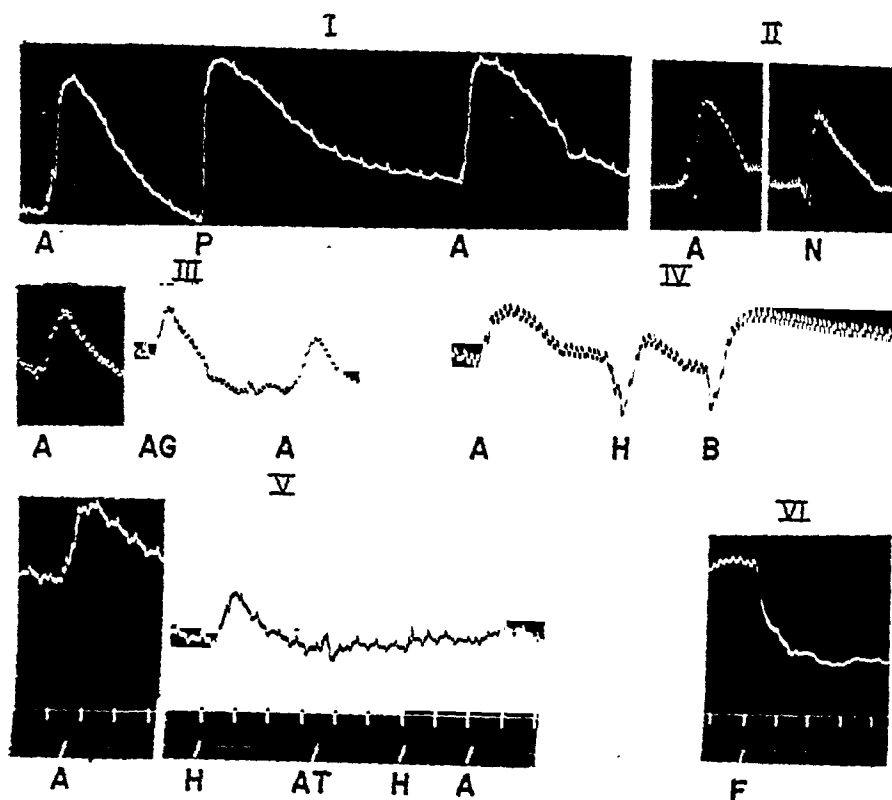


Fig. 4. Blood pressure records from six different cats. All injections through the coeliac cannula. I) 2  $\gamma$  adrenaline at A, 3 mg pyribenzamine at P. II) 4  $\gamma$  adrenaline at A, 5 mg neoantergan at N. III) 2  $\gamma$  adrenaline at A, 5 mg antergan at AG. IV) 1  $\gamma$  adrenaline at A, 2  $\gamma$  histamine at H, 2 mg benadryl at B. V) 2  $\gamma$  adrenaline at A, 0.4  $\gamma$  histamine at H, 10 mg antistine at AT. VI) 5 mg 929 F at F.

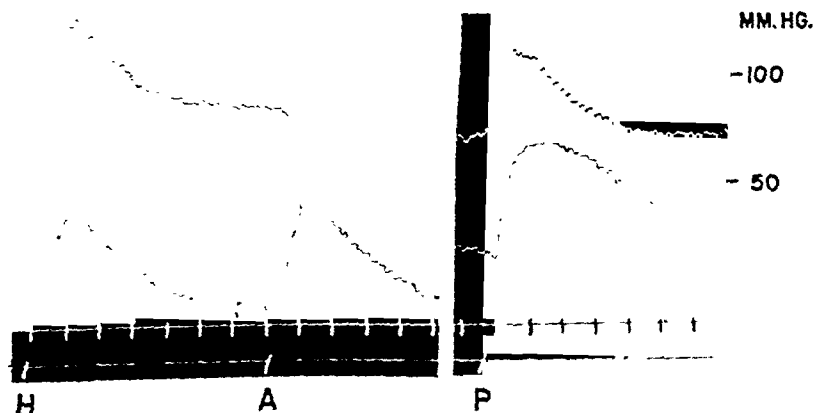


Fig. 5. Blood pressure (upper tracing) and denervated nictitating membrane (lower tracing) from a 2.5 kg cat. Ganglion coeliacum removed. Injections into the coeliac artery of 1  $\gamma$  histamine at H, 2  $\gamma$  adrenaline at A and 3 mg pyribenzamine at P.

an experiment with pyribenzamine which given through the coeliac cannula in a dose of 3 mg elicits a rise in blood pressure and contraction of the sensitized nictitating membrane after a latency of about 20 seconds. It is concluded that the antihistamine substances (except 929 F and antistine) cause an output of adrenaline from the cells of the suprarenal medulla. This view is supported by the following observations:

1. Intraarterial injections of these drugs are followed by effects on blood pressure and sensitized nictitating membrane which are characteristic for adrenaline.

2. The effects are obtained only if the drugs are given close arterially to the suprarenals, not if they are injected intravenously.

3. If the adrenals are removed the typical effect cannot be elicited. The effect is not a reflex one: it is obtained with denervated suprarenals.

In one experiment nicotine was injected into the coeliac artery causing an output of adrenaline. Repeated doses of nicotine were given until no response from the suprarenals was obtained with nicotine. Acetylcholine was now without effect whereas histamine still acted, although less than before nicotine. This effect has been described by SZCZYGIELSKI (1932). At this stage of the experiment pyribenzamine and neoantergan were found not to cause any output of adrenaline. From this experiment it is obvious that histamine and antihistamine drugs attack the medullary cells by means of different mechanisms. The action of the antihistamine drugs is more like that of choline. There is another similarity between the action of choline and of these drugs. After repeated doses of pyribenzamine, benadryl, antergan or neoantergan the response of the suprarenals usually becomes small. Choline shows a similar effect (GUTMANN 1932). It may be added that we have found that these big doses of the antihistamine compounds mentioned, which liberate adrenaline from the medullary cells, also stimulate the sympathetic postganglionic cells of the superior cervical ganglion, causing contraction of the nictitating membrane when injected intraarterially close to the ganglion.

### Discussion.

The remarkable ability of histamine to stimulate the release of the hormone of the suprarenal medulla into the blood stream which has been ascribed a physiological function by DALE and



his coworkers, is thus found to be antagonized by the so-called antihistamine compounds. This may suggest that histamine exerts its action on the adrenal cells through a mechanism of the same type as that through which it acts on smooth muscles and blood vessels but different from that acted upon in the gastric glands. This mechanism in the adrenals is apparently different from that through which acetylcholine, the mediator of preganglionic sympathetic impulses to the medulla (FELDBERG, MINZ and TSUDZIMURA 1934), liberates adrenaline: nicotine abolishes the effect of acetylcholine but not of histamine, whereas antihistamine compounds render the suprarenals insensitive to histamine but not to acetylcholine. It is thus possible specifically to antagonize the action of drugs on the secretory cells of the suprarenal medulla just as for instance on the smooth muscle cells of the intestine. It may be added that bile salts in our experiments liberate adrenaline after both nicotine and antihistamine substances just as *e. g.* barium ions act on the gut after atropine and neoantergan in low concentrations.

The ability of some antihistamine drugs to liberate adrenaline which to us first seemed highly suggestive and therefore was examined in some detail, does not seem related to the histamine antagonizing property as some of the drugs are devoid of this activity. Chemically our adrenaline liberators have in common the group  $-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH} (\text{CH}_3)_2$  linked to nitrogen (in anter-gan, neoantergan, and pyribenzamine) or to oxygen (in benadryl); 929 F and antistine lack this group.

This work has been supported by a grant from The Swedish Medical Research Council.

### Summary.

1. Antihistamine compounds (antergan, antistine, benadryl, neoantergan, 929 F and pyribenzamine) antagonize the action of histamine on the adrenal medulla.

2. This antagonism is specific. The adrenaline releasing effect of acetylcholine, KCl, or bile salts is not abolished by antihistamine drugs.

3. Antergan, benadryl, neoantergan and pyribenzamine injected into the central stump of the coeliac artery cause an output of adrenaline from the suprarenals.

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## **Liberation of Adrenaline from the Suprarenals Caused by Bile Salts.**

By

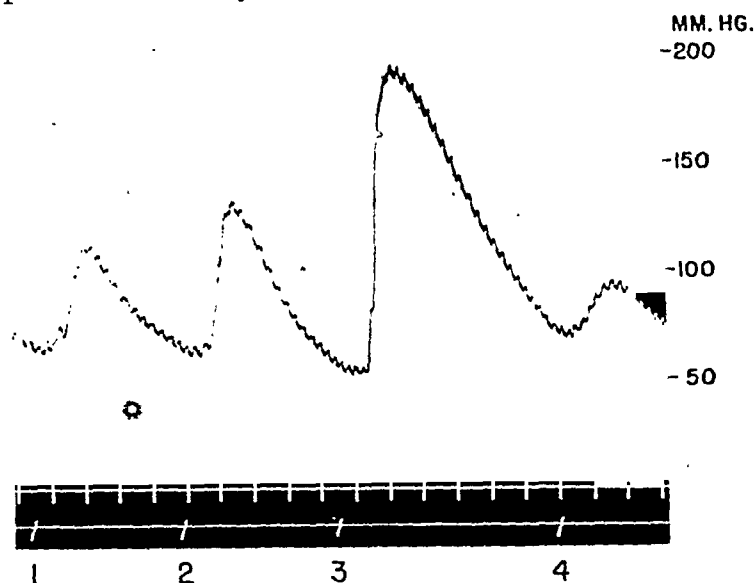
NILS EMMELIN and ANDERS MUREN.

Received 16 February 1949.

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In experiments described in a previous paper it was found that antihistamine compounds diminish or abolish the adrenaline release from the adrenal medulla elicited by histamine, whereas the release caused by acetylcholine is not affected (EMMELIN and MUREN 1949). Nicotine, on the other hand, is known to antagonize the adrenaline liberating action of acetylcholine but not, or to much less degree, that of histamine (SZCZYGIELSKI 1932). In our experiments we found it desirable to have at our disposal a substance which liberates adrenaline and which is antagonized neither by nicotine nor by antihistamine drugs; such a substance would enable us to ascertain that the suprarenals still contain and are able to give off adrenaline after treatment with nicotine and antihistamine compounds. *Lysolecithin* has been found to liberate adrenaline through its lytic action on the cells of the suprarenal medulla (FELDBERG 1940). This substance was not available, however; but we thought that bile salts might have a similar effect, and if this was found to be the case it seemed probable that an adrenaline release brought about in this drastic way might not be antagonized by nicotine or antihistamine drugs. LUDÁNY (1937) produced evidence that bile and bile salts injected intravenously cause an output of adrenaline reflexly, by lowering the blood pressure. Our experiments indicate that bile salts in addition can liberate adrenaline by a direct, probably lytic, action on the cells of the adrenal medulla.

The experiments were carried out on cats as described in our communication quoted above. The following bile salts were examined: sodium desoxycholate, sodium glycocholate, sodium taurocholate, and sodium dehydrocholate. The figure shows the effect of 0.5 mg of desoxycholate which produced a big rise in blood pressure when injected through the coeliac cannula. For



Record of blood pressure in a carotid artery from 2.7 kg cat in chloralose anaesthesia, eviscerated, vagi cut in the neck, coeliac ganglion removed. Injections into the central stump of the coeliac artery of 1) and 4) 3  $\gamma$  of adrenaline, 2) 0.4  $\gamma$  of histamine, 3) 0.5 mg of sodium desoxycholate. Time in minutes.

comparison the effects of 0.4  $\gamma$  of histamine and 3  $\gamma$  of adrenaline are shown. The response to adrenaline seemed somewhat diminished after desoxycholate; this is in agreement with an observation by RIES and STILL (1933), who found that bile salts decrease the sensitivity of the blood vessels to adrenaline. In all our experiments the suprarenals were denervated by removal of the coeliac ganglion. The effect of the bile salts is thus due to a direct action on the suprarenals. This can also be inferred from the fact that the pressor action of the bile salts was exerted even when the substances did not cause a previous fall in blood pressure, as is shown in the figure; and doses of bile salts which had a pressor action when given in the coeliac artery had a pure depressor effect when given intravenously. As was expected the bile salts were found to liberate adrenaline after nicotine; and even big doses of anti-histamine compounds, *e. g.* 30 mg of neoantergan

or 15 mg of pyribenzamine, given through the coeliac cannula, did not abolish the action of the bile salts on the suprarenals. When the different bile salts were compared it was found that the desoxycholate had the highest activity; 0.5 mg caused a big output of adrenaline. Glycocholate acted in doses of about 3 mg, taurocholate in doses of 8 mg. Dehydrocholate in a dose of 20 mg had no effect; as much as 50 mg produced a small effect only. Also with regard to hemolytic activity desoxycholate is known to have the highest, dehydrocholate the lowest activity of these bile salts (ROTHLIN and SCHALCH 1944) and it seems reasonable to suppose that the effect described here — an effect elicited by fairly big doses applied close arterially — is due to a lytic action of the bile salts on the cells of the adrenal medulla.

### Summary.

Bile salts, injected into the central stump of the coeliac artery in an eviscerated cat cause an output of adrenaline from the suprarenal medulla. This effect, which is not abolished by previous injections of nicotine or antihistamine compounds, is supposed to be due to a lytic action of the bile salts on the cells of the suprarenal medulla.

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From the Department of Physiology and the Clinic for Obstetrics  
and Gynaecology, University of Lund.

## A Simplified Method for Estimating the Histaminolytic Activity of Plasma in Pregnancy.<sup>1</sup>

By

FINN WICKSELL.

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It is well established that human blood in pregnancy contains an increased amount of a histamine-inactivating principle, an enzyme called *histaminase* or *diamine-oxidase*. This observation, first made by MARCOU et al. 1938, was confirmed by a great number of investigators (WERLE and EFFKEMANN 1940, ZELLER and BIRKHÄUSER 1940, AHLMARK 1944, VALENTIN 1945 and others). AHLMARK found that the determination of the histaminolytic activity of plasma could be used not only as a pregnancy test, but also for estimation of the age of a pregnancy. Further he found the values to be altered in certain obstetrical disorders of placental origin. In this connection it may be of interest to mention that the histaminase appearing in plasma in pregnancy seems to originate from the placenta (DANFORTH and GORHAM 1937, MARCOU et al. 1938, ZELLER et al. 1939, EFFKEMANN and WERLE 1940, AHLMARK 1944, ANREP et al. 1947). Through this work investigations on the histaminolytic activity in pregnancy became of clinical interest.

The methods for the determination of histaminolytic activity are chemical or biological. The chemical methods have been developed mainly by ZELLER and co-workers in a long series of papers

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<sup>1</sup> This paper forms part of the work presented to the Medical Faculty of the University of Lund as a thesis for a Medical Degree.

(for references see ZELLER 1940). These methods will give satisfactory results on sufficiently purified preparations of high activity, but when only low activities are present — which is the case in non-pregnant persons and in early pregnancy — the errors of the chemical methods will be considerable. By means of biological determination, however, it is possible to obtain rather accurate estimations even of low activities. These methods therefore seem to be preferable in pregnancy.

The methods for biological estimation of histaminolytic activity are all modifications of Best's original procedure (BEST 1929). The method of AHLMARK (1944) fulfils the high demands for accuracy and sensitivity required and is thus particularly well suited for these purposes.

This method is carried out in the following manner: 13—17 ml heparinized plasma is incubated at 37° C with 0.26 to 0.85 ml of a solution containing 30.4  $\gamma$  histamine base per ml in phosphate buffer. Samples are withdrawn at suitable intervals and treated according to CODE (1937) including 1) precipitation of the proteins with trichloroacetic acid, 2) boiling with HCl, 3) evaporation of the residue to dryness in vacuo, 4) removal of surplus acid with abs. alcohol, 5) taking up of the dry residue in distilled water and neutralization. The extracts thus obtained are assayed on a piece of ileum from a guinea-pig. The histaminolytic power is expressed as the quantity of histamine base (in  $\gamma$ ) inactivated by 1 ml plasma in the course of one hour ( $\gamma$ /ml/h). According to AHLMARK his method has an average error of 7—16 %. If differences in the histamine content of the samples of less than 20 % are considered insignificant and if the incubation time is not more than 24 hours the method will permit determination of histaminolytic activities as low as 0.05  $\gamma$ /ml/h. It may be concluded that with this method it is possible to detect low histaminolytic activities and that the accuracy of the method is quite satisfactory in view of the very considerable changes in histaminolytic power of plasma in the different stages of human pregnancy.

But AHLMARK's method has a distinct disadvantage. It is rather complicated to carry out and this limits its value for clinical purposes. For every histaminase determination about four plasma samples have to be passed through Code's extraction procedure before they can be assayed on the isolated intestine of a guinea-pig. It would be advantageous if this lengthy treatment could be simplified or avoided without undue loss of accuracy. The experiments of this paper were carried out for this purpose.

When my experiments were almost completed, ANREP, BARSOUM and IBRAHIM (1947) published a method aiming at a similar

simplification. In their experiments a sample of 0.9 ml serum is mixed with 1  $\gamma$  histamine base and incubated at 37° C for 30 minutes. It is then diluted with 5 ml Tyrode's solution and heated to 80° C. Finally the sample is assayed on the guinea-pig's gut. The result is given in per cent inactivation over 30 minutes. This procedure will be further discussed later in this paper.

## Experimental.

1. *Assay of the samples after boiling only, instead of extraction.* It is well known that the histamine content of blood or plasma can not be assayed directly on the isolated guinea-pig's ileum since there are other substances present — proteins, adenosine compounds and others — which interfere with the test (BARSOUM and GADDUM 1935, CODE 1937, AHLMARK 1944). My experiments confirm this. Plasma concentrations in the test bath of  $\frac{1}{2}$ —1 p.c. or more were found to make the assay unreliable. The plasma decreases the histamine sensitivity of the gut. On repeated administration of plasma the inhibitory effect on the gut increases and soon becomes complete. If, however, the plasma is first heated to boiling point some of the disturbing substances are inactivated. According to my experiments it is now possible to increase the plasma concentration to about 4 % without the assay being materially disturbed. A plasma sample can thus be determined directly if it is diluted 2.5 times with an inert salt solution and if the volume introduced is less than 10 p.c. of the volume of the test bath, provided, of course, that its histamine content is sufficiently high to permit determination.

The histaminolytic activity of plasma from pregnant women near term was estimated in the following way. Heparinized<sup>1</sup> blood was centrifuged and plasma withdrawn. The plasma, a histamine solution containing 25.  $\gamma$ /ml<sup>2</sup> and Tyrode's solution were placed separately in a water bath at 37°. After about 15 minutes 1 ml plasma and 5  $\gamma$  histamine were mixed and made up to 25 ml with Tyrode's solution giving an initial histamine concentration of 0.20  $\gamma$ /ml in the mixture. (The histamine content of the plasma, about 20  $\gamma$ /l, is negligible in this connection.) The mixture was

<sup>1</sup> Tricresol-free heparine was used in these experiments.

<sup>2</sup> All the histamine figures in this paper are expressed as base unless otherwise stated.



well shaken and again placed in the incubator. A sample with a volume of 2—4 ml was withdrawn immediately after mixing and then samples were taken every 15 minutes. The samples were quickly heated to boiling point in order to destroy the enzyme and thus arrest histaminolysis. They were then kept untouched at 4° C in the refrigerator until the final assay was performed on the isolated guinea-pig's ileum. The result of an experiment of this type is seen in fig. 1.

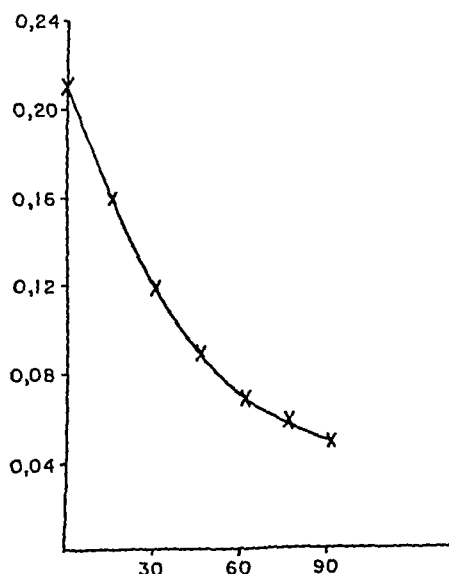


Fig. 1. Inactivation of histamine by pregnancy plasma.

Abscissa: time in minutes.

Ordinate: undecomposed histamine in  $\gamma$ /ml of the mixture.

Initial concentration 0.20  $\gamma$ /ml.

As a control the same experiment was repeated with the difference that the mixture was heated to boiling point immediately at the beginning of the incubation. None of these samples had lost any of their activity after 24 hours.

As is seen from fig. 1 the histamine inactivation takes place at a nearly constant rate until about half of the histamine is destroyed. The reaction then gradually becomes slower. This is a common characteristic of enzyme-substrate reactions. Concerning the histaminase-histamine reaction it was demonstrated as early as 1929 by BEST.

2. *Comparison with extraction procedure.* It is of interest to compare the results given by the simplified method that has just been described with the results when the samples are previously treated according to CODE (1937). The experiments of this type were performed in the following way. Two portions of pregnancy plasma each 1.0 ml, were mixed with 5  $\gamma$  histamine and diluted to give an initial histamine concentration of 0.20 or 0.25  $\gamma$ /ml. They were incubated at 37° C as described above. Samples were withdrawn from both mixtures at 0, 30, 60, 90 and 120 minutes. The samples from one tube were boiled and tested directly. The samples from the second tube were precipitated in trichloro-acetic acid and passed further on through CODE's extraction procedure. The results of two experiments of this type

are shown in fig. 2, where the inactivation is recorded until about half of the histamine activity is lost.

It will be seen from fig. 2 that the histamine figures obtained with the extraction method are somewhat lower than those obtained through simplified assay. Presumably this depends on

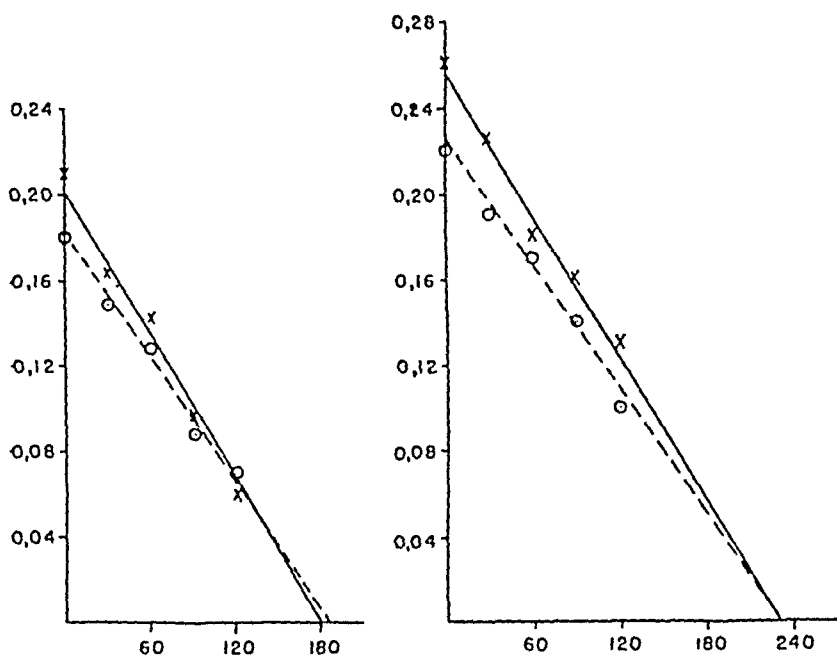


Fig. 2. Comparison between samples obtained by the simplified method and the extraction procedure.

Abscissa: time in minutes.

Ordinate: undecomposed histamine in  $\gamma$ /ml in the mixture.

Initial concentration in the left figure 0.20  $\gamma$ /ml, in the right figure 0.25  $\gamma$ /ml.

— x boiled samples.

- - - o extracted samples.

small losses of histamine during the chemical treatment of the samples. But it is noteworthy that both inactivation curves tend towards the same point on the abscissa, *i. e.* in reality they have the same inactivation rate.

In table 1 a comparison is made between values obtained by AHLMARK with his method and the results obtained with the simplified method described in this paper. AHLMARK's values are extracted from figures 12 and 13 in his monograph on this subject (1944). My own figures were obtained from 29 determinations on 27 healthy, pregnant women. As demonstrated in table 1, the values obtained with the simplified method were largely of

Table 1.

*Histaminolytic power of plasma determined with the simplified method and compared with Ahlmark's method.*

Age of pregnancy	Ahlmark's figures $\gamma/\text{ml/h}$	Own figures $\gamma/\text{ml/h}$
6 weeks .....	0.001—0.015	0.01
7 » .....	0.006—0.02	0.02
8 » .....	0.007—0.08	0.02
		0.02
9 » .....	0.015—0.19	0.06
10 » .....	0.05 —0.40	0.10
		0.11
11 » .....	0.12 —0.70	0.39
		0.14
		0.12
		0.17
12 » .....	0.20 —1.0	0.18
		0.48
		0.25
4 months .....	0.42 —2.0	0.71
		0.70
5 » .....	0.9 —5.0	2.0
		2.0
		1.4
6 » .....	1.3 —5.3	3.0
		1.9
		1.6
7—10 » .....	1.6 —5.4	3.3
		1.5
		2.5
		2.6
		2.2
		3.5
		1.7

the same order as those obtained by AHLMARK with the extraction method.

3. *Principle of calculating the histaminolytic activity.* Like AHLMARK (1944) I have chosen to express the histaminolytic activity as the quantity of histamine (in  $\gamma$ ) which is inactivated by one ml of plasma in one hour ( $\gamma/\text{ml/h}$ ). For the exact calculation of the activity it would be necessary to deduce the formula of the inactivation rate. But I have found it more convenient for practical purposes to calculate the activity as a function of the time necessary for destruction of 50 p.c. of the added histamine. However, to calculate the exact time at which there is 50 p.c. inactivation would require a comparatively large number of observations. It will now be demonstrated that it is possible to base an estimation of the histaminolytic activity on a single observation, without losing much in accuracy.

In fig. 3. I have calculated an inactivation curve based on ten experiments. (To obtain this curve each experiment was graphically represented, the time of 50 p.c. inactivation serving as a common unit for the incubation times, and a joint curve was calculated.) It is seen from this curve that the inactivation rate

does not decrease materially during the first 60 p.c. of the inactivation. In the figure three lines have been drawn from the histamine value at zero time through the points on the curve showing 30, 50 and 60 p.c. inactivation respectively. As can be read from the abscissa a determination of the histaminolytic activity, based on 60 p.c. inactivation (straight thin line in fig. 3) differs by about 10 p.c.

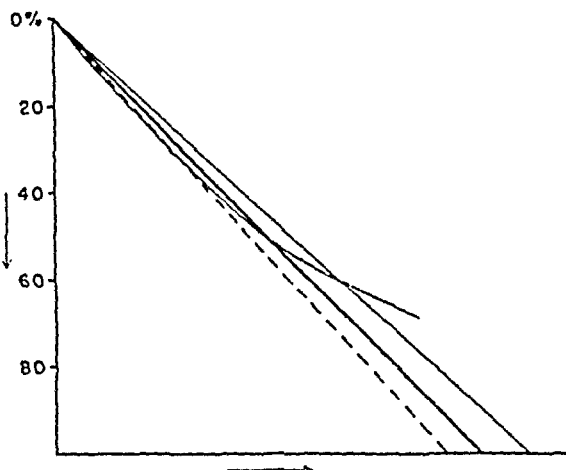


Fig. 3. Histamine inactivation curve (curved thin line).

Abcissa: time.

Ordinate: per cent histamine inactivation.

For details see text.

from what is obtained if the estimation is based on 50 p.c. inactivation (straight heavy line) and a calculation based on 30 p.c. inactivation (straight dotted line) differs by only 9 p.c. from the 50 p.c. value. It is obvious from fig. 3 that the calculation of the histaminolytic activity without materially increasing the error may be based on any point of the curve provided that the inactivation is less than 60 p.c. But here another factor must be taken into consideration, the error of the assay on the guinea-pig's ileum. AHLMARK calculates this error as 3.4 p.c. I find it safe to count with an average error of 5 p.c. That makes an error of  $\pm 4$  p.c. if the calculation is based on a value obtained at 60 p.c. inactivation, the error is  $\pm 5$  p.c. at 50 p.c. inactivation,  $\pm 12$  p.c. at 30 p.c. inactivation and at 10 p.c. inactivation it is as high as  $\pm 55$  p.c. My calculations of the histaminolytic power are based on values obtained between 30–60 p.c. histamine inactivation.

4. *The errors of the method.* The errors are of three kinds. A. *Measuring errors.* These have not been calculated in detail but a

rough estimation shows that they will not exceed 5 p.c. B. *The inactivation proceeds at a gradually decreasing rate.* If the calculation is carried out between 30 and 60 p.c. inactivation, this error may be graphically determined to be  $\pm 10$  p.c. (See page 365.) C. *The accuracy of the histamine determination.* As stated above I have counted with an average error of 5 p.c. of the assay on the ileum and this makes an error of 4 p.c. at an inactivation of 60 p.c. At 30 p.c. inactivation it makes an error of 12 p.c.

These factors give the following error of the method: at 60 p.c. inactivation  $\sqrt{5^2 + 10^2 + 4^2} = \pm 11.9$  p.c. and at 30 p.c. inactivation  $\sqrt{5^2 + 10^2 + 12^2} = \pm 16.4$  p.c. The method thus has an error of 12–17 p.c. This may be considered as sufficiently accurate, particularly if the enormous increase of the histaminolytic power of plasma during human pregnancy is taken into consideration.

5. *The technique of the method.* It remains to give a description of the carrying out of the determinations. Plasma, histamine solution (25  $\gamma$ /ml) and Tyrode's solution are allowed to stand separately for about 15 minutes in an incubator at 37° C in order to acquire this temperature. These components are mixed in proportions that will be described below and the incubation commenced. One sample is withdrawn immediately (0 hour). Other samples are taken at intervals, which are chosen so that the incubation time is double that of the previous sample. With some experience it is possible roughly to predict the histaminolytic activity. I have found it advantageous to choose intervals so that the sample in which I expect about 50 p.c. inactivation will be the fourth. If, for example, half the histamine activity is expected to be lost after 6 hours the following incubation times would be used: 0, 1½, 3, 6, 12, 24 hours. With these intervals one of the samples will show an inactivation somewhere between 30 and 60 p.c. As a rule 6–8 samples are withdrawn, each of them having a volume of 2–3 ml. They are immediately heated to boiling point and kept at + 4° C until the final assay takes place on the isolated guinea-pig's ileum.

The gut preparation is made in the usual way. A guinea-pig is killed by a blow on its head, the abdominal cavity is opened and a 15–20 cm piece of the lower ileum is freed from the mesentery. The gut is thoroughly washed out with Tyrode's solution. It is then suspended in Tyrode, with O<sub>2</sub> containing 1 p.c. CO<sub>2</sub> continuously bubbling through. The temperature of the bath is kept

at 32° C. After 15—30 minutes the gut begins to perform violent contractions. A suitable piece is suspended in a test bath containing 2.5 ml. No atropine is used. The assay, which is carried out at a temperature of 32° C is now begun. The first thing to do is to check that the 0-hour sample contains the calculated amount of histamine. For this purpose I use a standard solution of histamine, containing 0.05—0.20  $\gamma$ /ml. It is relatively simple to find out which of the other samples contains 70—40 p.c. of the histamine content of the 0-hour sample. The histamine content of the former sample is now determined as accurately as possible, using the 0-hour sample as a standard. The histaminolytic power of the plasma is then calculated from the formula

$$\text{Histaminolytic power} = \frac{V(H_0 - H_x)}{v \cdot T} \gamma/\text{ml/h.}$$

In this formula  $v$  is the volume of the plasma in ml,  $V$  is the volume of the mixture in ml,  $H_0$  is the initial histamine concentration of the mixture in  $\gamma$ /ml,  $H_x$  is the histamine content (in  $\gamma$ /ml) after incubation during  $T$  hours. For example: 1 ml plasma is incubated with 5  $\gamma$  histamine and Tyrode's solution making a volume of 25 ml. This gives an initial histamine content of 0.2  $\gamma$ /ml. After 12 hours incubation the mixture contains 0.12  $\gamma$  histamine per ml. The equation gives

$$\text{Histaminolytic power} = \frac{25 (0.20 - 0.12)}{1 \cdot 12} = 0.17 \gamma/\text{ml/h.}$$

In my determinations I have used the following mixtures: 1. When the histaminolytic power is stronger than 0.1  $\gamma$ /ml/h: 1.0 ml plasma, 5.0  $\gamma$  histamine and Tyrode's solution giving the mixture a final volume of 25 ml. 2. When the histaminolytic power is less than 0.1  $\gamma$ /ml/h: 2.0 ml plasma, 1.0  $\gamma$  histamine and Tyrode's solution ad 20 ml. Using this mixture and incubation times up to 24 hours a histaminolytic power of 0.006  $\gamma$ /ml/h may be detected.

### Discussion.

The method described in this paper has been worked out for plasma, but gives substantially similar results if serum is used. I have used plasma because it has often been desirable to investigate the histamine content of plasma simultaneously. The method will also be applicable to some tissue extracts and to lymph, as

will be demonstrated in a subsequent paper. Total blood may not be used, however, since the corpuscles contain substances which interfere with the assay on the isolated guinea-pig's gut.

Recently ANREP, BARSOUM and IBRAHIM (1947) have published a method which in some respects resembles the method described here. They state that differences between 0 and 10 p.c. inactivation are too small to be detected with certainty. That means that the weakest histaminolytic activity that can be determined with this method is 0.22  $\gamma$ /ml/h (histamine expressed as base). For values between 30 and 60 p.c. inactivation the method will work well. With increasing histaminolytic activities the figures will then have an increasing error. The strongest activity that can be determined with this method (100 p.c. inactivation) would correspond to 2.2  $\gamma$ /ml/h. The values mentioned above are found between the eleventh and eighteenth weeks of human pregnancy. In other periods of pregnancy the method of ANREP, BARSOUM and IBRAHIM will not be practicable.

With the method described in this paper it is possible to detect histaminolytic activities as low as 0.006  $\gamma$ /ml/h with an error of about 15 p.c. There is no upper limit of the activities which can be determined. The author has used this method in about 100 cases of various disorders during pregnancy. This material is still being collected and will be published in a separate communication. The method described here does not differ fundamentally from Ahlmark's method and is to be considered as a modification of the latter. However, it is easier to perform since the lengthy extraction procedure is omitted and only small quantities of plasma or serum will be required (1—2 ml instead of 13—17 ml in Ahlmark's method). For this reason it should prove of value in obstetrical practice. It has also proved useful for determinations of histaminolytic activity carried out in experiments described in other papers of this volume.

### Summary.

A modification of Ahlmark's method for the determination of histaminolytic activity of plasma during pregnancy is described. It is demonstrated that the lengthy and drastic chemical treatment of the plasma samples may be omitted.

In principle the method described consists of three steps:

1. Incubation at 37° C of a mixture containing plasma, histamine and Tyrode's solution.
2. Withdrawal of samples at certain intervals and arrestment of histaminolysis by heating these samples to boiling point.
3. Biological assay of the undecomposed histamine on isolated gut from a guinea-pig.

Histaminolytic activities as low as 0.006  $\gamma$ /ml/h may be detected with this method. The error is 12—17 per cent.

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From the Department of Physiology, University of Lund.

# **The Strong Histaminolytic Activity of Lymph and its Bearing on the Distribution of Histamine between Lymph and Plasma in Dogs.**

By

ARNE CARLSTEN, GEORG KAHLSON and FINN WICKSELL.

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In studies on the liberation of chemical agents, instrumental in eliciting and modifying normal functional activities, such as adrenaline, acetylcholine and histamine, such agents have been traced principally in venous effluents. The failure in this laboratory to demonstrate the occurrence of histamine in venous effluents during reactive hyperaemia and muscular tetanus, where histamine is alleged by some investigators to be liberated, led us to search for histamine in the lymph. The lymph is closer to the tissue cells which are thought to liberate histamine, and it thus seemed likely that liberated histamine should accumulate in greater concentrations in the lymph than in the blood. As a preliminary to the investigations planned under this assumption we investigated the distribution of histamine between lymph and plasma under conditions where the plasma was known to contain excess histamine. In the course of these studies we detected that the lymph is protected against the accumulation of active histamine much more efficiently than is the blood. We also observed that the lymph, unlike plasma, is very active in destroying histamine.

## **Histamine in Lymph and Plasma after Injection of Histamine and Administration of Histamine-Liberators.**

1. *Normal lymph and plasma.* All experiments reported in the present communication were made on non pregnant dogs anaes-

thetized with nembutal. Lymph was collected from a cannula inserted into the thoracic duct after opening the chest. Full technical details are given in another paper included in this volume. Extracts for histamine determinations were prepared by ultrafiltration and by the extraction procedure, also fully referred to in our second paper (CARLSTEN, KAHLSON and WICKSELL, 1949). The biological assay was performed on the guinea-



Fig. 1. Piece of guinea-pig's ileum suspended in a 4 ml capacity bath. Time signal every minute. Added at mark 1: 0.01  $\gamma$  Hi; 2: 0.02  $\gamma$  Hi; 3: 0.3 ml U. F. of normal plasma; 4: 0.012  $\gamma$  Hi; 5: 0.3 ml U. F. normal lymph; 6: neoantergan  $1: 5 \cdot 10^6$  in the bath; 7: 0.15 ml U. F. normal plasma; 8: 0.15 ml U. F. normal lymph.

pig's ileum and the cat's blood pressure. The histamine antagonist neoantergan was used to strengthen the identification of histamine.

In dogs, contrary to guinea-pigs, rats and rabbits, the histamine activity of plasma is low. In dogs we never obtained figures exceeding 65  $\gamma$  per liter, thus confirming the findings of EMMELIN (1945). This activity, however, was not regularly completely annulled by neoantergan. For this reason, and since precise information on the histamine activity of normal plasma is not essential for the purpose of the present study, we feel satisfied with the general statement that in the dogs referred to here, the histamine activity of normal plasma in every instance was less than 65  $\gamma$ /l. In the present work we have not conclusively determined to what proportion the histamine activity in normal plasma, referred to in

the tables, is actually due to histamine; for this reason the figures for normal "plasma histamine" are given in brackets.

In our hands extracts and ultrafiltrates (U.F.) from normal canine lymph never contained histamine activity in detectable amounts. As the methods employed enabled the demonstration of histamine in concentrations as low as 5 to 10  $\gamma$  per liter and we used especially sensitive guts for the assay of lymph extracts, it is justifiable to state that the histamine activity of lymph, if present at all, is lower than 5 to 10  $\gamma$  per liter. It may be mentioned here that normal lymph occasionally contains a gut contracting agent other than histamine, which is irrelevant to the problem investigated here. (Fig. 1.) The nature of this agent is discussed in another communication in this volume. — Incidentally we may record here that native lymph frequently contains a factor or factors which considerably increases the histamine sensitivity of the guinea-pig's gut, unlike plasma which depresses the sensitivity.

2. *Observations with injection of histamine.* In 9 dogs (6 to 31 kg in weight) histamine was administered by slow steady infusion into a vein in doses ranging from 25  $\gamma$  to 400  $\gamma$  per minute during 15 to 90 minutes. In each of 5 dogs (8 to 26 kg) histamine was administered in ten subcutaneous depots in total doses of 1 to 2 mg per kg body weight. Blood pressure was recorded from a carotid artery and the rate of lymph flow registered with a method described in our following paper. (Fig. 2.) Before injections of histamine, control samples of lymph and plasma were obtained. Other samples of blood were collected from a brachial artery just before the end of infusion and after subcutaneous injections when the fall in blood pressure was manifest. Corresponding lymph samples were collected during the entire course of infusion, respectively during the period of fall in blood pressure subsequent to subcutaneous injection. The results are summarized in tables 1 and 2.

Table 1 shows that at the end of prolonged infusions of histamine in the doses employed the histamine concentration of plasma may reach extremely high levels, as already reported by EMMELIN, KAHLSON and WICKSELL (1941) for shorter periods of infusion of smaller quantities than used here. It is a surprising fact that the plasma in these experiments is carrying excess histamine in copious quantities without a corresponding rise in the lymph. With the exception of dog no. 9 the lymph did not contain excess histamine in detectable amounts. (Fig. 3.) In this dog histamine

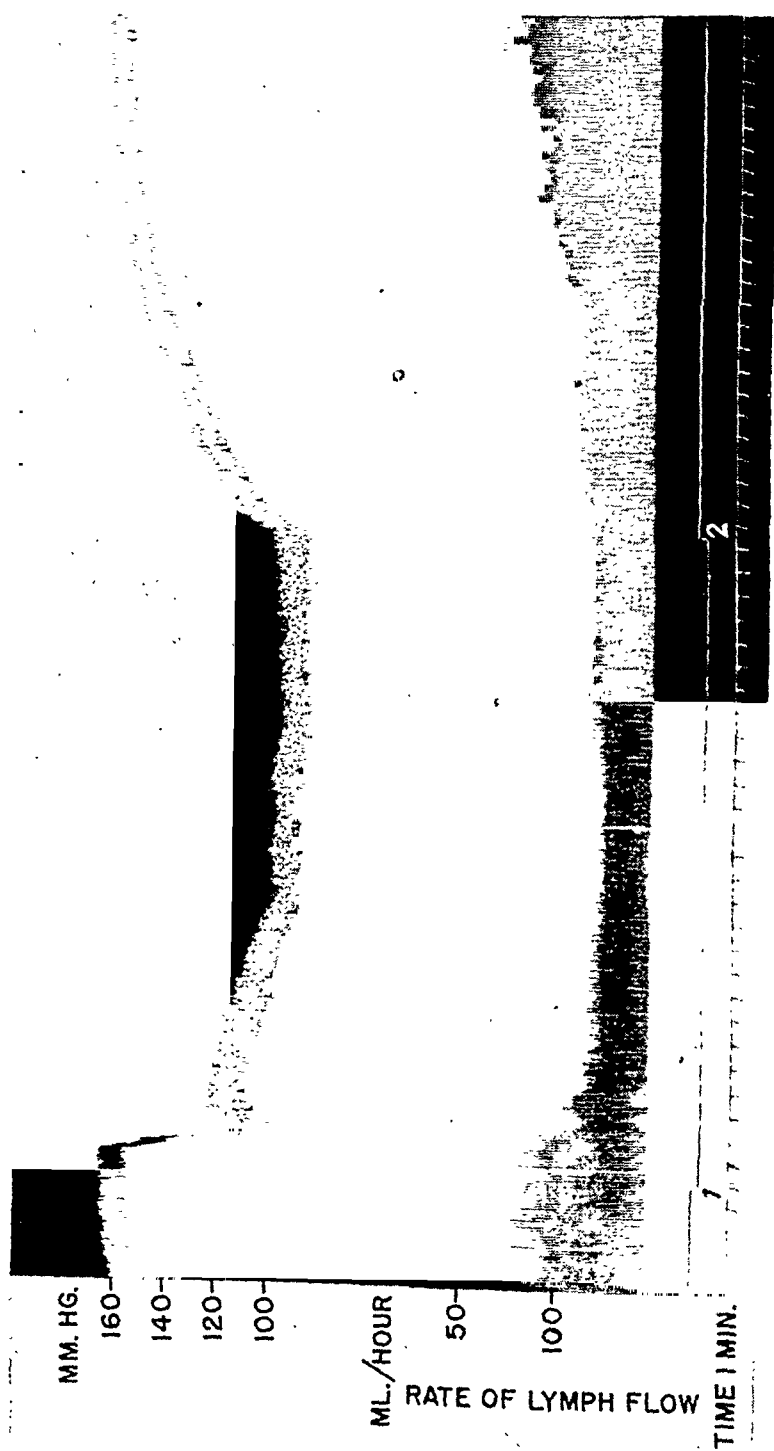


Fig. 2. Dog 15 kg, nembutal. Between marks 1 and 2 intravenous infusion of histamine 0.2 mg per minute. In recording rate of lymph flow the ordinates are inversely proportional to rate of flow.

Table 1.

*Intravenous Infusion of Histamine.*

No. of dog	Weight of dog kg	Dosage of histamine $\gamma/\text{min.}$	Duration of infusion minutes	Fall of blood pressure mm/Hg	Lymph flow ml/h		Hist. in plasma $\gamma/\text{l}$		Hist. in lymph $\gamma/\text{l}$	
					Before inf.	During inf.	Before inf.	During inf.	Before inf.	During inf.
1	6	40	90	20	—	—	(24)	580	<10	<10
2	10	75	60	60	14	42	(50)	1200	<10	<10
3	28	100	15	10	—	—	(10)	120	<6	<10
4	14	100	60	25	23	74	(11)	120	<3	<3
5	15	100 200	55 30	30 65	21	60 95	(65)	130	<10	<10
6	12	200	30	30	9	94	(9)	800	<2	<2
7	12	140	30	30	5	18	(4)	650	<4	<4
8	31	25	55	25	48	43	(40)	80	<10	<10
9	15	100 200 300 400	20 20 20 40	20 50 75 75	—	— — — —	(3)	— — — 700	<3	— — — 70

Table 2.

*Subcutaneous Injection of Histamine.*

No. of dog	Weight of dog kg	Dosage of histamine mg/kg	Fall of blood pressure mm/Hg	Lymph flow ml/hour		Hist. in plasma $\gamma/\text{l}$		Hist. in lymph $\gamma/\text{l}$	
				Before inj.	After inj.	Before inj.	After inj.	Before inj.	After inj.
14	26	2	50	50	75	(6)	140	<4	
15	20	2	55	29	53	(3)	40	<2	<4
16	8	2	65	—	—	(2)	75	<2	<2
17	24	2	100	37	86	(5)	85	<5	<5
18	19	1	30	50	94		170	<3	<3

was infused in increasing dosage over 100 minutes until the death of the animal induced by the histamine. It is an interesting point that in this dog the histamine destroying activity of the lymph was far below the average figure as shown in table 6.

Table 2 illustrates that on subcutaneous injections of large doses of histamine excess histamine appears in the plasma but not in the lymph.

3. *Histamine liberators.* It is well established that curare (ALAM, ANREP, BARSOUM, TALAAT and WIENINGER 1939) and trypsin (ROCHAE SILVA 1939, 1946) cause the liberation of histamine from different types of tissue cells. In a series of experiments we administered these substances<sup>1</sup> intravenously in quantities which elicited a profound and prolonged fall in blood pressure and caused a considerable increase in the plasma histamine, at least in the curare experiments. The results are given in the tables 3 and 4.

Confirming the results of ANREP and his co-workers, curare proved a very powerful histamine liberator. In this type of experiment histamine, diffusing from the site of liberation onwards to the small vessels, passes through the interstitial fluid, and seems to have the opportunity of accumulating in especially high concentration in the lymph. Contrary to expectation, however, no excess histamine occurs in the lymph emerging from the thoracic duct. — Trypsin, in our hands, was a less efficient histamine liberator than curare. Only in three of six dogs did trypsin in doses which caused a profound fall in blood pressure induce a rise in plasma histamine. After trypsin no excess histamine could be detected in the lymph.

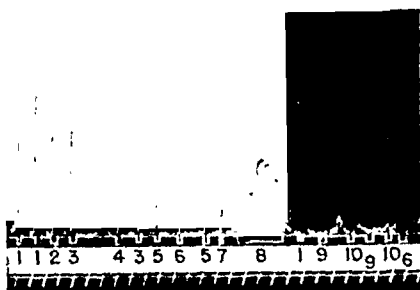


Fig. 3. Guinea-pig's ileum. Added to the bath at 1: 0.03  $\gamma$  Hi; 2: 0.05 ml U. F. plasma after intravenous Hi-infusion; 3: 0.02  $\gamma$  Hi; 4: 0.3 ml U. F. lymph after intravenous Hi; 5: 0.01  $\gamma$  Hi; 6: 0.2 ml U. F. of lymph after intravenous Hi; 7: 0.005  $\gamma$  Hi; 8: neoantergan 1:  $5 \cdot 10^6$  in the bath; 9: 0.04  $\gamma$  Hi; 10: 0.1 ml U.F. plasma after intravenous Hi.

<sup>1</sup> We are indebted to Dr. PEHR EDMAN and Professor K. LINDERSTROM-LANG who gave us pure preparations of trypsin.

Table 3.

*Curare.*

No. of dog	Weight kg	Dose Curare Intocostrin Squibb	Blood pressure mm/Hg before and after curare	Plasma UF $\gamma/l$		Lymph UF $\gamma/l$	
				Before	After	Before	After
22	12	6 mg	135—80	(28)	120	<10	—
25	25	7.5 mg	125—90	(10)	27	<4	<4
26	24	6 mg	150—80	(8)	35	<10	<10
		6 mg			30	<10	<10
27	11	12 mg	175—55	(5)		<4	<4
31	22	6 mg	160—75	(33)	190	<4	<4

Table 4.

*Trypsin.*

Dog no	Weight kg	Dose Trypsin mg/kg	Blood pressure mm/Hg before and after trypsin	Plasma UF $\gamma/l$		Lymph UF $\gamma/l$	
				Before	After	Before	After
7	31	3	130—75	(40)	90	<10	<10
11	13	8	100—40	(25)	120	<10	<10
12	20	8	80—35	(65)	45	<10	<10
14	20	10	145—65	(22)	27	<2	<3
20	14	10	135—40	(7)	21	<4	<4
21	13	10	110—50	(5)	5	<5	<5

### The Histamine Destroying Activity of Lymph.

At an early stage of this study it appeared desirable to correlate the histamine side of the present work with concomitant determinations of the histaminolytic activity of lymph and plasma. This seemed especially imperative for lymph, the histaminolytic activity of which has not been investigated previously.

The method used for determining histaminolytic activity is that used by WICKSELL (1949) for plasma, which is described in a

separate communication. This method proved useful also for lymph and extracts of lymph nodes. In the following tables this activity is expressed in  $\gamma$  histamine destroyed by one ml lymph

Table 5.

Hi-ase in plasma $\gamma$ Hi/ml/h	Hi-ase in in lymph $\gamma$ Hi/ml/h	Ratio Hi-ase Lymph/ Plasma
0.16	2.0	12.5
<0.05	3.0	<60
<0.05	0.28	> 5.6
<0.04	2.5	> 62.5
0.10	0.19	1.9
0.05	1.2	24
0.07	5.7	81.4
<0.04	0.17	> 4.3
0.06	0.59	9.8
<0.04	—	—
—	0.25	—
—	0.20	—
—	0.77	—
—	1.1	—
—	0.50	—
—	3.9	—
Average: <0.07	1.50	> 29.1

or plasma in one hour, and for the sake of brevity referred to as histaminase.

The histaminolytic activity of plasma was determined in 10 dogs, that of lymph in 15 dogs. In the plasma the figures varied from less than 0.04 to 0.16  $\gamma$ /ml/hour with a mean value of < 0.07.



Table 6.  
*Hi-Infusion i. v.*

Dog no.	Hi-ase in plasma $\gamma$ Hi/ml/h		Hi-ase in lymph $\gamma$ Hi/ml/h	
	Before inf.	After inf.	Before inf.	After inf.
1 .....	<0.04	<0.05		
2 .....	<0.05	0.11	3.0	2.9
3 .....	<0.05	<0.05	0.28	0.59
4 .....	<0.04	<0.04	2.5	0.36
5 .....	0.10	<0.05	0.19	0.15
6 .....	0.05	0.08	1.2	1.1
7 .....	0.07	0.08	5.7	4.7
8 .....	<0.04	<0.04	0.17	0.16
9 .....	0.06	0.06	0.59	0.45
34 .....	0.16		2.0	

The corresponding figures in lymph are 0.17 to 5.7  $\gamma$ /ml/hour with a mean value of 1.50. The figures are given in table 5. The average ratio of histaminolytic activity of lymph and plasma is  $> 30$ .

It seemed of interest to investigate whether the histaminolytic activity of lymph and plasma changed in response to excess histamine derived from injected or liberated histamine. In the series of experiments represented in table 1 where large doses of histamine were infused intravenously during 15 to 100 minutes the histamine-destroying activity of lymph and plasma were determined before and at the end of infusion. The results are summarized in table 6. No significant changes occur.

In the experiments represented in table 2 in which the histamine content of lymph and plasma was determined before and after subcutaneous injections of histamine the histaminolytic activity of lymph was estimated concomitantly. The results are given in table 7; they are in accord with the observations illustrated in table 6.

Similar determinations were done in some of the dogs, re-

ferred to in the tables 3 and 4, in which curare or trypsin was administered. The histaminolytic power of the lymph is stated in table 8. As with histamine injections the histamine liberators caused no significant change.

**Table 7.**  
*Histaminase.*  
Histamine Subcutaneously.

Dog no.	H-ase in lymph	
	Before	After
14 .....	0.25	0.50
15 .....	0.20	0.14
16 .....	0.77	0.78
17 .....	1.1	—
	—	—

In 4 dogs various lymph glands were examined for their histaminolytic power. The excised glands were minced and ground with quartz powder and Tyrode's solution. After separation in the centrifuge the histaminolytic activity of the supernatant extract was determined with the routine method as already referred to. The results are summarized in table 9.

*In vivo experiments on histamine destruction by the lymph.* In a special series of experiments, as a corollary to our in vitro de-

**Table 8.**  
*Histaminase in Lymph.*

Dog no.	Curare		Trypsin	
	Before	After	Before	After
26 .....	0.06	0.04		
27 .....	0.50	0.50		
11 .....			3.9	4.0
7 .....			0.17	0.16

Table 9.  
*Histaminase in Lymph Nodes.*  
 $\gamma/\text{Hi}/\text{gr}/\text{h.}$

Dog no.	32	33	34	35
Mesenteric nodes .....	1.7	2.9	6.1	10.5
Bronchial nodes .....	2.3	4.7	1.0	1.5
Inguinal and popliteal nodes .	1.8	3.8	2.3	3.2
Hypogastric nodes .....	—	—	2.0	1.3
Cervical nodes .....	—	3.5	1.1	—
Portal nodes .....	1.6	3.4	—	—

terminations of the histamine-destroying activity, we examined in vivo the rate of histamine inactivation by the lymph. The following procedure was employed: a cannula was inserted into the central stump of a mesenterial or femoral lymphatic trunc. Into this cannula histamine was infused in doses ranging from 1.5  $\gamma$  to 100  $\gamma$  per min. for 10 to 20 min. Lymph was collected from the thoracic duct before and during the infusion of histamine and examined for its histamine content. The results are summarized in table 10.

As seen in table 10 the intralymphatic infusion of large doses of histamine is accompanied by a slight fall in blood pressure. This

Table 10.

Dog no.	Weight kg	Fall in blood pressure mm/Hg	Lymph flow		Hist. in plasma $\gamma/\text{l}$		Hist. injected during 20 min. in $\gamma$	Hist. recovered in duct lymph $\gamma$
			Before inf.	During inf.	Before inf.	During inf.		
10	28	10	50	128	(8)	28	2,000	600
13	24	15	37	59	(65)	70	2,000	170
19	19	0 0	50	78 60	(3)	3 4	30 100	3.5 5.5
23	24	0 0 0 30	56	104 96 84 87	(6)	6 — 8 60	36 144 600 4,800	0 0 3.9 290

indicates that in our preparation some histamine escapes into the the general circulation. The communication concerned is probably principally established by the main right lymphatic duct, which was not dissected for ligation. The amount of histamine thus eluding recovery from the cannula in the thoracic duct can be calculated from experiments of EMMELIN, KAHLSON and WICKSELL (1941) who demonstrated that in the dog, slow intravenous infusion of 0.3  $\gamma$  histamine per kg/min. causes a fall in blood pressure of approximately 10 mm Hg. The calculation of the amount of recovered and destroyed histamine was done under the assumption that very roughly 10% of the intralymphatically infused histamine escapes into the circulation by side channels. On these premises and with due attention to the changes in the rate of

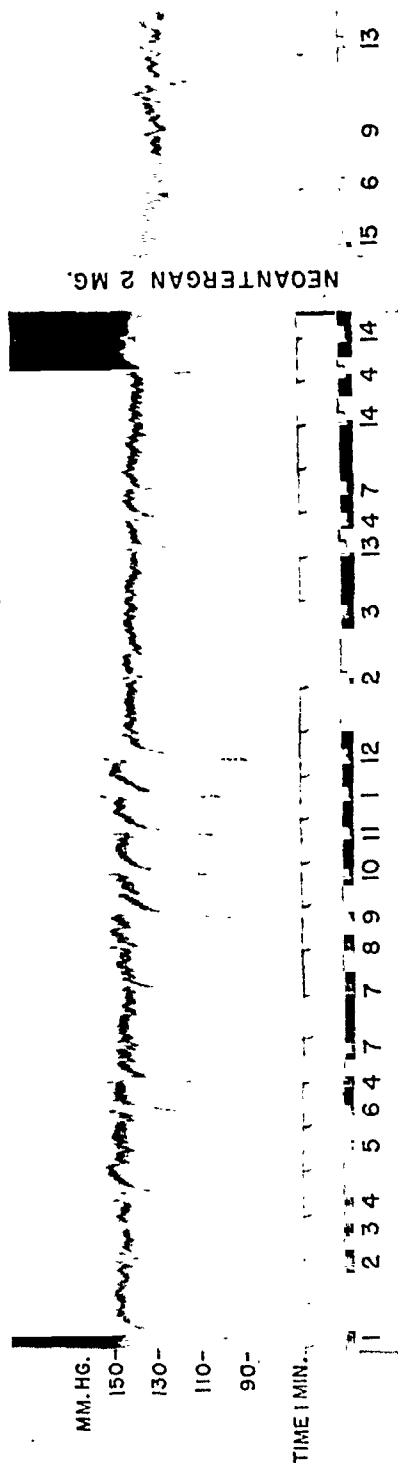


Fig. 4. Cat, 2.1 kg, eviscerated, chloralose. Injected into a vein at 1: 0.25  $\gamma$  Hi; 2: 1 ml U. F. normal plasma; 3: 1 ml U. F. normal lymph; 4: 0.1  $\gamma$  Hi; 5: 1 ml U. F. plasma after intralymphatic infusion of 25  $\gamma$ /min. Hi; 6: 1.0 ml U. F. lymph corresponding to 5; 7: 0.05  $\gamma$  Hi; 8: 1.0 ml U. F. plasma after intralymphatic Hi 100  $\gamma$ /min.; 9: 1.0 ml U. F. lymph corresponding to 8; 10: 0.15  $\gamma$  Hi; 11: 0.2  $\gamma$  Hi; 12: 0.35  $\gamma$  Hi; 13: 0.5 ml U. F. plasma after subcutaneous Hi; 14: 0.5 ml U. F. lymph corresponding to 13; 15: 0.3  $\gamma$  Hi; neoantergan 2 mg was given intravenously between marks 14 and 15.

lymph flow caused by the histamine infusion it can be very roughly estimated that a large proportion of the infused histamine was destroyed during the passage through the restricted system of lymphatics perfused. (Fig. 4.) With the smallest doses no histamine was recovered from the thoracic cannula. — Whatever the error of this estimate, it seems certain that *in vivo* the lymph is capable of inactivating large quantities of histamine very rapidly.

### Discussion.

High histaminolytic activity in the blood has previously been observed in man during pregnancy. Evidence has been provided to show that the placenta is the source of the histaminolytic agent in the maternal blood (AHLMARK 1944, ANREP, BARSOUM and IBRAHIM 1947). It is demonstrated in the present investigation on non pregnant dogs that the lymph is unexpectedly rich in histaminolytic activity, the source of which obviously is some other than the placenta. Neither are the lymphatic glands likely to represent centers of production, as the histamine inactivating capacity of lymph nodes, at least *in vitro*, is not conspicuously high.

In the *in vitro* experiments the mean values of observations in 15 dogs for the histaminolytic activity of lymph and plasma is 1.5  $\gamma$  and  $< 0.07 \gamma/\text{ml/h}$  respectively. The average ratio of activity lymph/plasma determined for each of nine dogs was found to be  $> 30$ .

Our estimate of the rate of *in vivo* destruction is impaired by gross errors. However, the experiments suggest that *in vivo* histamine inactivation by the lymph proceeds very rapidly, probably much more rapidly than *in vitro*.

It is a remarkable fact that in the dog the histamine concentration of plasma can be raised to any level compatible with survival without the appearance of excess histamine in the lymph. The lymph, and possibly the interstitial fluid, seems to provide an efficient means of protecting the tissue cells from the action of excess histamine.

It has been reported that histaminolytic activity is absent in the plasma of dogs (ANREP, BARSOUM, IBRAHIM 1947). These investigators employed a method which is less adapted for the demonstration of the low activity normally present in canine plasma. In our experiments the plasma *in vitro* regularly destroyed

histamine, although at a very low rate. We have confirmed the finding of the workers in Cairo that histaminolysis is not altered by injections of histamine, as suggested by MARCOU (1938, 1939) and others.

Attempts to reveal the source of the histaminolytic activity in the lymph and to assess more accurately this activity *in vivo* are in progress in this laboratory.

### Summary.

In anaesthetised dogs the histamine-destroying activity of lymph and plasma and the distribution of histamine between lymph and plasma under various conditions were determined with the following results.

1. The lymph is very potent in destroying histamine. *In vitro* the histaminolytic activity of lymph is on the average  $> 30$  times greater than of plasma. *In vivo* intralymphatically administered histamine is inactivated at a very high rate.

2. Lymph normally does not contain histamine in detectable quantities. The histamine concentration of plasma can be raised to very high levels by histamine injections and by histamine liberators without the appearance of the slightest trace of excess histamine in the lymph.

This work was aided by a grant to one of the authors (G. K.) from the Swedish Medical Research Council.

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From the Department of Physiology, University of Lund.

## **Accumulation in the Canine Lymph During Reactive Hyperaemia of a Substance Contracting the Guinea Pig's Gut Which is Not Histamine.**

By

ARNE CARLSTEN, GEORG KAHLSON and FINN WICKSELL.

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Attempts in this laboratory to demonstrate, during reactive hyperaemia in man, dogs and cats, the liberation of histamine from the ischaemic tissues into the blood draining them, have been uniformly unsuccessful (EMMELIN, KAHLSON and WICKSELL 1941). Using potent histamine antagonists, such as benadryl and neoantergan, it was demonstrated that the extent of reactive hyperaemia in dogs and cats was not reduced by rendering the small blood vessels completely insensitive to even considerable doses of histamine (EMMELIN and EMMELIN 1947; FOLKOW, HAEGER and KAHLSON 1948). Previous workers studying the liberation of histamine during reactive hyperaemia have examined the blood or plasma emerging from the tissues in which the blood flow was arrested or restricted; this was done on the assumption that the liberated histamine diffuses from the deficiently supplied cells into the tissue spaces and from there to the small blood vessels. If histamine or other active agents are actually released during reactive hyperaemia, from the inadequately supplied cells, such agents should primarily be accumulated within the tissue spaces. For this reason we thought it essential to examine the lymph draining tissues the blood supply of which was restricted. — The relevant literature has been reviewed quite recently by GADDUM (1948).

## Experimental.

*Preparation of the dog.* All experiments were made on dogs anaesthetised with "nembutal" intravenously, omitting initial ether. The object was to collect lymph predominantly from the caudal part in which the blood flow was restricted. The thoracic duct was dissected after resection of the second and third left ribs. A cannula was inserted into the duct at the height of the cardiac base. To prevent clotting the cannula and the attached plastic tubing was covered by silicon (JAQUES, FIDLAR, FELDSTED and MACDONALL 1946) and wetted with a 5 % solution of heparine. In order to exclude lymphatic channels other than those draining the hind legs and the lower part of the body truncus the following steps were undertaken: arterial branches to the entire gastrointestinal tract, the liver, the pancreas and the spleen were obstructed by ligatures. The abdominal aorta was dissected in the region closely caudal to the renal arteries, precautions being taken not to damage the lymphatic trunks which run beside the aorta. In order to eliminate anastomotic arteries from deeper regions an écraseur was applied to the region where the aorta had been freed for the obstructing clamp, special care being taken not to cause injury to deeper lymphatics. To promote the flow of lymph the hind limbs were flexed and stretched passively at a rate of 80 jerks per minute; the device was analogous to that used by MC CARREL (1939) for passive motion of the head and perfusion of the nasopharynx.

The rate of lymph flow through the cannula inserted into the thoracic duct was recorded continuously with a technique used as a routine in this laboratory, devised by CLEMENTZ and RYBERG and described by them in a separate communication included in this volume. The time intervals between two successive drops was recorded using a modified "Ordinatenschreiber" by FLEISCH (1930); with this device the height of the registered ordinate is proportional to the time interval, *i. e.* inversed proportional to the rate of flow.

In order to evoke reactive hyperaemia the abdominal aorta was obstructed with a clamp. The circulation distal to the clamp was arrested for a period of 15 min. followed by 5 min. of free circulation. Such cycles of consecutive arrest and free passage were repeated five to ten times.

The lungs were ventilated with a pump of the Starling type. The body temperature was kept at approximately 37° C. Physiological saline, 50 ml every hour, was administered subcutaneously. The blood pressure was recorded from a cannula inserted into a carotid artery.

The major proportion of the lymph emerging from the cannula originated from regions subjected to arrested circulation. Fig. 1 is a typical experiment performed to elucidate this point. At the mark 1 passive motion of the legs was initiated, causing an increase in the rate of lymph flow from approximately 20 to 75 ml per hour; the legs were passively moved during the entire course of the experiment. The interval 3—4 indicates the period during which the aorta was obstructed. As is seen in fig. 1, arrest of the circulation in the regions distal to the



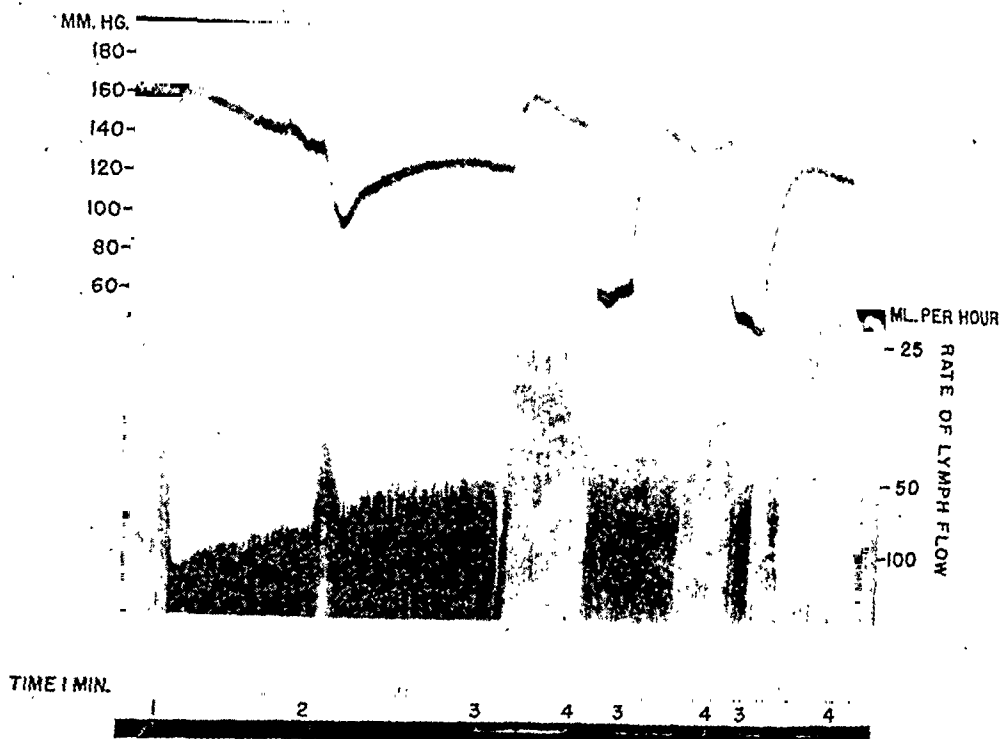


Fig. 1. Dog 20 kg body weight, nembutal. The numbers indicate: 1: passive motion of the hind legs initiated; 2: 1 ml Intocostrin-T intravenously; 3: aorta obstructed; 4: obstruction released.

clamp causes a diminution in the rate of lymph flow from approximately 50 to less than 25 ml per hour. In this actual experiment, as well as in a few others, curare ("intocostrin-T" Squibb) was injected in doses just sufficient to eliminate spontaneous respiratory movements which interfered with the proper recording of lymph flow. There are no objections to this procedure, since the histamine liberated by curare does not appear in the lymph. This is fully discussed in our preceding paper (CARLSTEN, KAHLSON and WICKSELL 1949).

In the present study considerable quantities of lymph were required. For technical reasons such quantities could not be collected by cannulating a group of individual lesser abdominal lymphatic trunks. Large quantities of duct lymph diluted with lymph from regions with free blood supply were more useful than inadequate amounts of plain lymph from the region rendered ischaemic.

*Preparation of the lymph for assay.* The lymph was collected in a glass vessel to which heparine had been added. The individual samples of lymph were treated as follows:

1. The native lymph was ultrafiltrated in the centrifuge as described by REHBERG (1943) and practiced by EMMELIN (1945) for estimating plasma histamine. For comparison, blood plasma was obtained and ultrafiltrated by the same technique.

2. In most of the experiments a proportion of every individual sample of lymph was used for determination of its histamine destroying capacity. The method hereby employed is fully described in a paper by WICKSELL (1949) included in this volume.

3. In some experiments the lymph was extracted for its histamine activity using the method of BARSOUM and GADDUM (1935) as simplified by CODE (1937).

*Biological assay.* The ultrafiltrates and the extracts were tested on the guinea-pig's ileum and the blood pressure of the eviscerated cat under chloralose. As an aid in the identification of histamine activity the histamine antagonist neoantergan was used.

## Results.

A. *Histamine destroying activity.* This activity was determined by a method described by WICKSELL (1949). Samples of lymph were collected firstly at the very beginning of the experiment before the viscera were functionally eliminated by arterial ligatures, secondly after "evisceration" and thirdly after repeated periods of reactive hyperaemia (R. H.). The results are summarized in table 1. The histaminolytic power of lymph in the dog does not change significantly in reactive hyperaemia. It is interesting to note, that evisceration of the type performed here, in some instances was followed by a considerable increase in the histaminolytic activity of the lymph. This change occurs rapidly within less than an hour. In two of the first experiments of table 1, where the figures related to evisceration are missing, the increase in histaminolytic power obviously is a sequel of evisceration and not of reactive hyperaemia.

B. *Histamine in reactive hyperaemia.* As discussed in the preceding paper (CARLSTEN, KAHLSON and WICKSELL 1949) lymph

Table 1.

*Histaminolytic power of lymph from dogs.*

Dog nr.	Control	After evisceration	During R. H.
1 .....	0.10	—	1.0
2 .....	< 0.04	—	1.8
3 .....	0.44	—	0.48
4 .....	0.17	2.2	2.2
5 .....	0.08	5.6	6.0
6 .....	0.42	0.45	0.62
7 .....	1.3	1.5	1.5
8 .....	—	1.1	1.2
9 .....	4.5	3.9	3.9
10 .....	0.03	0.12	0.10

from the aenesthetised dog, under otherwise normal conditions, does not contain detectable quantities of histamine. In 16 dogs subjected to arrest of the circulation by obstruction of the aorta, histamine uniformly could not be detected in the lymph. As far as histamine is concerned lymph collected during reactive hyperaemia does not differ perceptibly from normal lymph. This is

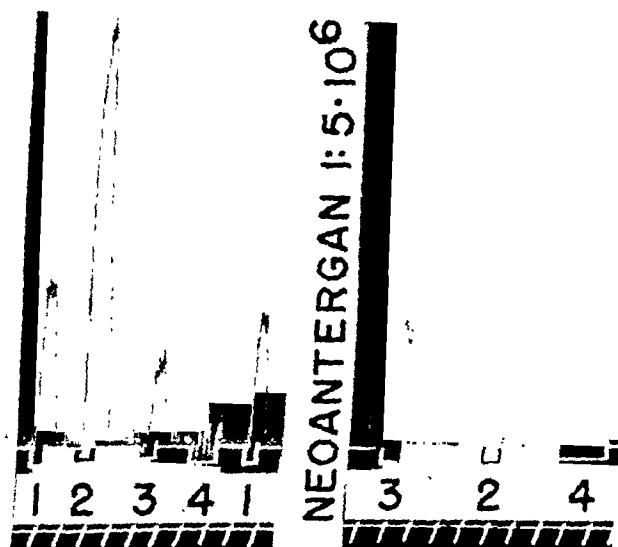


Fig. 2. Piece of guinea-pig's ileum suspended in a bath 4 ml in capacity. Added to the bath at 1: 0.01  $\gamma$  histamine base; 2: 0.02  $\gamma$  histamine; 3: 0.3 ml ultrafiltrate (U. F.) of normal plasma; 4: 0.3 ml of normal lymph (U. F.).

true even for samples collected during later periods of reactive hyperaemia preceded by several instances of circulatory arrest and not contaminated by previously discharged lymph.

In a few experiments venous blood was collected from the femoral vein during reactive hyperaemia. To satisfy the conditions requisite according to ANREP and his associates (1944) for the demonstration of excess histamine in plasma, the blood flow was controlled, allowing only a very restricted rate of flow after releasing the obstruction. In none of these experiments did excess histamine appear in the venous plasma during reactive hyperaemia. This applies to ultrafiltrates as well as to extracts of plasma.

C. *Active agents other than histamine.* On examining more closely the action of ultrafiltrates on the guinea-pig's ileum we discovered that they, under certain conditions, contain one, or possibly more than one, substance which causes the gut to contract. This type of activity is only irregular and then not conspicuously ob-

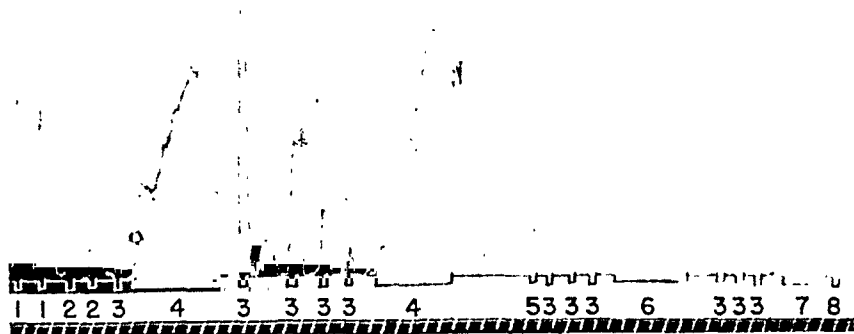


Fig. 3. Guinea-pig's ileum. Added to the bath at 1: 0.035  $\gamma$  histamine; 2: 0.03  $\gamma$  histamine; 3: 0.01  $\gamma$  histamine; 4: 0.3 ml U. F. of lymph collected during reactive hyperaemia (R. H.); 5: 0.005  $\gamma$  histamine; 6: 0.3 ml U. F. of plasma during R. H.; 7: neoantergan  $1:5 \times 10^6$ ; 8: 0.04  $\gamma$  histamine.

served in normal lymph. (Fig. 2.) However, this activity appears regularly in the lymph when the animal has been subjected to repeated periods of circulatory arrest. In some instances the activity also appears in ultrafiltrates of plasma where, however it is present in a much lower degree than in the corresponding lymph (Fig. 3).

This activity differs from that of histamine in the following points:

1. The onset of contraction occurs with a latency which is unmistakeably longer than with histamine. In the experiment illustrated by the figures 3 and 4 the latencies vary from 9 to 20 seconds, whereas on adding histamine, choline, acetylcholine or potassium ions to the bath the recording lever starts to ascend with a delay of only 2 to 4 seconds.

2. The ascent and descent of contraction is slow as compared with histamine and the other substances mentioned.

3. A piece of guinea-pig's gut in contact with histamine contracts and relaxes when left in contact with histamine. With the other type of activity referred to here, contraction of the gut is maintained for many minutes when the active ultrafiltrate is left in contact with the gut. On replacing the content of the bath by pure Tyrode's solution, after-contractions are frequently observed in response to the washing out, and relaxation proceeds only very slowly, contrary to the steep and uncomplicated relaxation after histamine.

4. When the gut, under the influence of active ultrafiltrates from lymph, has been subjected to this non-histamine type of activity, it acquires an increased sensitivity to histamine as

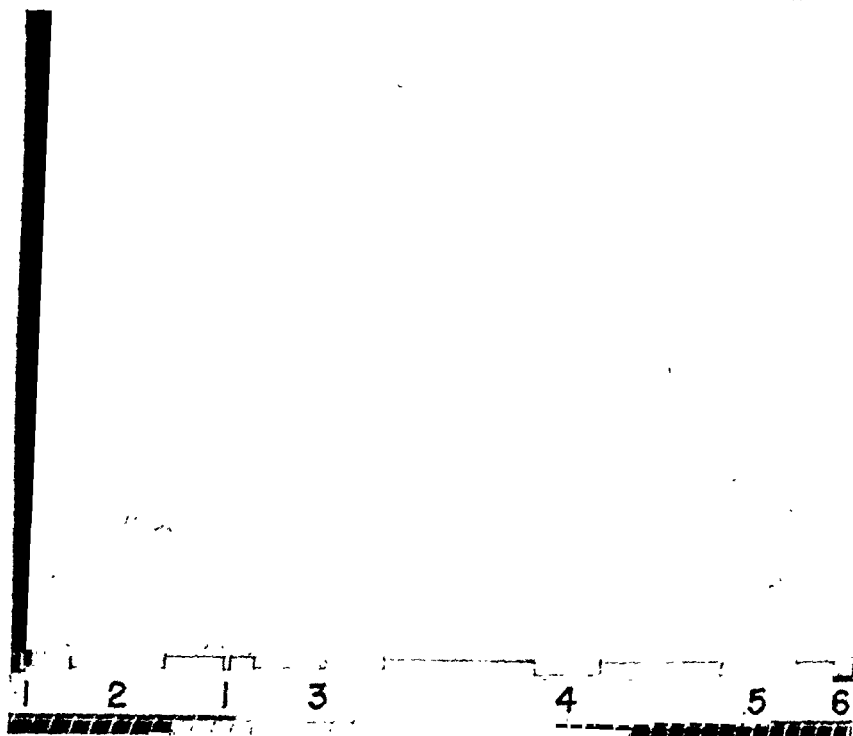


Fig. 4. Guinea-pig's ileum, continued from fig. 3, the gut treated with neoantergan added to the bath at 1: 0.04  $\gamma$  histamine; 2: 0.3 ml U. F. of plasma during R. H.; 3: 0.3 ml U. F. of lymph during R. H.; 4: 0.15 U. F. of lymph during R. H.; 5: 0.15 ml U. F. of plasma during R. H.; 6: 0.05  $\gamma$  histamine.

exemplified in fig. 3, mark 3. Under the influence of ultrafiltrates from lymph collected during reactive hyperaemia the histamine sensitivity of the gut is often more than doubled; this sensitization recedes rather quickly after washing the gut as illustrated in fig. 3, indicated by the consecutive marks 3.

5. The activity persisted in guts treated with neoantergan in the concentration 1:  $10^7$  and 1:  $5 \times 10^6$ . This concentration renders the gut completely insensitive to histamine in doses which are relevant in our observations, as demonstrated in fig. 3.

The ultrafiltrates containing gut contracting activity of the non-histamine type were also tested for their effect on the cat's blood pressure. In a few experiments the rate of blood flow through the hind legs of the cat was measured and the changes caused by injection of the ultrafiltrate into the central stump of the inferior mesenteric artery observed. In none of these experiments did injection of 1 ml of ultrafiltrate containing gut-contracting material cause changes in blood pressure or rate of blood flow through the limbs.

We have observed that in the dog ultrafiltrates from normal lymph and plasma not infrequently contain a material, non-histamine in nature, which causes a contraction of the gut. The contraction resembles that elicited by histamine, except for the unmistakeably longer latency and for the fact that it persists after neoantergan. These features are exemplified in fig. 2. This latter type of activity is studied closely by us in experiments in progress which we intend to report in a forthcoming communication in this journal.

### Comments.

At the beginning of these experiments it was thought that examination of the histamine content of lymph would throw new light on the concern of histamine in the vasodilatation of reactive hyperaemia. In the course of this study we discovered that the lymph in dogs has a high capacity to destroy histamine as compared with the weak histaminolytic power of plasma. Analogous with what is recorded about the occurrence of acetylcholine in effluents, in the absence of eserine, it thus did not seem very likely that relatively small quantities of histamine, if they were liberated during the normal functional activity of reactive hyperaemia, should withstand the destruction by the lymph to emerge in the lymphatic effluent. Our experiments have only substantiated this supposition. On the other hand, GEBAUER-FUELNEGG, DRAGSTEDT and MULLENIX (1932) are credited with having demonstrated the presence of histamine in the lymph flowing from the dog's liver in anaphylactic shock. The bearing of their report on our problem can not easily be assessed, since in anaphylaxis histamine is liberated in large quantities and nothing is known about the histaminolytic activity of liver lymph.

We also failed to detect an increase of histamine in the venous blood emerging from tissues with restricted circulation during reactive hyperaemia. This confirms earlier observations recorded by this laboratory and adds to the evidence, in so far as work with ultrafiltrates of plasma might be safer than work with the traditional extracts.

In the introduction we referred to observations from this laboratory showing that vasodilatation in reactive hyperaemia is not abolished or even reduced in extent by rendering the blood vessels completely insensitive to injected histamine. This was not con-

sidered as conclusive evidence against histamine in reactive hyperaemia. There remains the possibility that under the influence of inadequate oxygen supply, histamine might be internally liberated in the very walls of the blood vessels and that these, under the influence of histamine antagonists, would be insensitive to what DALE (1948) refers to as "extrinsic" histamine and yet respond to "intrinsic" histamine. Whatever the mechanism of histamine liberation in reactive hyperaemia, it seems to us that the successful demonstration of histamine in lymphatic and venous effluents in this type of normal activity will have to await the discovery of some means to protect the physiologically liberated histamine from destruction on its passage with the draining effluents.

It is well established that the histaminolytic power of plasma varies widely in one and the same individual, notably in human pregnancy, directed by mechanisms and functions which are only poorly understood. The changes observed so far occur slowly in the course of weeks and months as extensively discussed by AHLMARK (1944) and by ANREP and his co-workers (1947). Justified not by analogy, but by our observation with evisceration where the histaminolytic power of the lymph increased rapidly, and by suggestions from different quarters that increased histaminolytic activity might be mobilized as a protective means against excess histamine (*e. g.* ADDARIO 1937), we thought it of some interest to examine the histaminolytic power of lymph during reactive hyperaemia. There was no change as compared with lymph collected before the first period of hyperaemia.

The direction of our effort mainly towards the lymph, instead of the plasma, led to the detection, in reactive hyperaemia, of a gut contracting agent, or possibly agents, with pharmacological effects different from histamine. Ultrafiltrates rich in this factor, when injected into the circulation, had, however, no effect on the cat's blood pressure. Obviously this does not exclude, that the smooth muscle contracting agent, if present in sufficiently high concentration, may effect the tone of the blood vessels. In experiments which we will report later we have demonstrated that the accumulation of this agent in the lymph is governed by one common condition, namely anoxia. We are also engaged in studying vascular and other effects of concentrated extracts of lymph potent in contracting the guinea-pig's gut. A discussion of the possible relationship of our observations to the "slowly-reacting sub-

stances" discovered by FELDBERG, KELLAWAY and TRETHEWIE (reviewed by KELLAWAY 1947) will have to be postponed awaiting more detailed knowledge on our part.

### Summary.

In reactive hyperaemia on anaesthetized dogs the following observations were made:

1. Ultrafiltrates and extracts from lymph emerging from regions during reactive hyperaemia after restriction of the circulation did not contain histamine in higher concentration than corresponding samples obtained during unrestricted circulation; in both instances the histamine content of the lymph was below that which can be detected by available methods.

2. Reactive hyperaemia did not cause an increase in the histamine concentration of venous plasma emerging from regions subjected to circulatory arrest.

3. The histaminolytic activity of lymph was not changed by reactive hyperaemia.

4. After repeated periods of arrest of the circulation and subsequent reactive hyperaemia an agent which contracts the guinea-pig's ileum accumulated in the lymph. Some pharmacological properties of this agent are described.

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From the Department of Physiology and the Obstetrical and  
Gynaecological Clinic, University of Lund.

## Observations on Histamine and Histaminolysis in Pregnancy.<sup>1</sup>

By

FINN WICKSELL.

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It was first suggested by DALE and LAIDLAW (1910) that histamine might be concerned in pregnancy. They demonstrated that histamine injected subcutaneously into a cat in late pregnancy caused strong periodic contractions of the uterus. These contractions were of a tonic type and had no expulsive value; they were likely to cause separation of the placenta and asphyxiation of the foetuses, which were expelled dead by the normal action of the uterus when the direct effect of the injected histamine on that organ had receded. DALE and LAIDLAW suggested that very small doses of histamine, "by simply increasing the excitability of the muscle, would add power to the coordinate contractions produced by nerve impulses or by the automatic rhythm of the organ". This hypothesis seemed to gain experimental support by an observation by HUKUDA (1940), who reported an increase in the histamine content of blood and urine in man during pregnancy. He suggested that this increase might be due to an inhibition of the histaminolysis, caused by sex hormones, particularly the oestrogens. He gave, however, only incomplete information of the methods employed in estimating the histamine content, which makes it difficult to evaluate his results. They do not seem to be in agreement with an earlier statement

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<sup>1</sup> This paper forms part of a thesis presented to the Medical Faculty, University of Lund, for obtaining the degree of M. D.

by MARCOU et al. (1938) who claimed that the histamine content of blood is below normal a few hours before and after delivery. HUKUDA's observations on urine confirm those of UNGAR and DUBOIS (1937), who recorded the presence of histamine in the urine of pregnant women, but were unable to detect any in non-pregnant persons. Later KAPPELLER-ADLER (1941) reported the presence of increased amounts of histamine in the urine of pregnant women; specially high figures were found in cases of toxæmia of pregnancy. In view of the contradictory results from the experiments on the histamine content of blood in pregnancy it seemed desirable to repeat these experiments, especially in connection with delivery where histamine according to the hypothesis of DALE and LAIDLAW might be involved. Also it seemed more interesting to study plasma than total blood. The literature quoted above supports the view that the histamine content of urine is increased in pregnancy; but there are no experiments on the histamine in urine in connection with delivery. Part 1 of this investigation deals with the histamine content of plasma and urine in parturition. It may be mentioned already here that these experiments did not indicate any changes in the histamine concentration either of plasma or of urine.

There is, however, another experimental finding reported in the literature which indirectly centers interest on histamine in pregnancy. It has been shown that human blood in pregnancy contains an increased amount of a histamine inactivating principle, an enzyme called histaminase. It has also been called diamineoxidase because it decomposes not only histamine but also certain diamines; some triamines are also attacked (ZELLER 1938, ZELLER, SCHÄR and STAEHLIN 1939). Of these substances only histamine has pronounced physiological effects and it is therefore reasonable to assume that the activity of the enzyme in the body is directed against histamine. The histaminase of the blood seems to originate from the placenta which is very rich in this enzyme (DANFORTH and GORHAM 1937, MARCOU et al. 1938, ZELLER et al. 1939, EFFKEMANN and WERLE 1940, AHLMARK 1944, ANREP et al. 1947). According to SWANBERG (1948) the formation probably takes place in the decidua, which shows particularly strong activity. Nothing is known as to the possible function of this enzyme appearing in blood and placenta in pregnancy. It might be hypothesized that the placental histaminase offers some kind of protection of mother or foetus against histamine. I have tried

to find out if the activity of this enzyme in the placenta manifests itself in differences in the histamine content of maternal or foetal blood or in a decrease in histamine content of foetal blood during its passage through the placenta. These experiments form Part 2 of this paper.

Another possibility is that the protective action against histamine is exerted by the blood histaminase and that the placenta merely serves as the site of formation of this enzyme. In the experiments accounted for in Part 3 I have investigated if the histaminolytic activity of blood is reflected in an altered sensitivity of the organism to injected histamine.

It should be emphasized that very high histaminolytic activity in pregnancy occurs only in human beings. For instance AHLMARK (1944) reported that the increase in plasma may amount to 1,000—2,000 times the non-pregnant activity. In other mammals there is no or only a very moderate increase in histaminolytic activity. This, of course, limits very much the possibility to attack the problems concerning histamine metabolism and histaminolytic activity in pregnancy experimentally.

## Methods.

*A. Methods for the determination of histamine.* All histamine assays were carried out on the isolated guinea-pig's ileum in the ordinary way. The assay on the gut did generally not permit satisfactory determination of a histamine content less than 10  $\gamma$ /l. Only with exceptionally sensitive intestine preparations was it possible to estimate histamine equivalents as low as 2  $\gamma$ /l although the error is apt to be greater than with higher values. In my tables such low values are given in brackets and classified as being less than 10  $\gamma$ /l. Prior to the assay the samples were treated in one of the following ways. *Blood* was prepared according to the method of BARSOUM and GADDUM (1935) as modified by CODE (1937). *Plasma* was treated either in the same way as blood or ultrafiltered according to EMMELIN (1945 a).

For the extraction according to CODE I have found it advantageous to employ the following slight modifications. Boiling with hydrochloric acid was carried out in a waterbath instead of a sandbath. This minimizes the risk of overheating the samples and less supervision is required. During the process of alcohol evaporation there is considerable danger of losing histamine since this substance is easily destroyed in an alkaline medium. It is therefore essential that this step is carried out as rapidly as possible. Evaporation of alcohol was performed under suction with condenser on a boiling waterbath and required about two minutes, this procedure being repeated three times with a total time

requirement of 5–7 minutes. With these precautions the loss of histamine during the entire extraction procedure did not exceed 20 p. c., as evaluated by addition of known quantities of histamine to plasma samples. Some plasma extracts prepared according to CODE exerted a strong gut-contracting activity, which was not inhibited by neoan-tergan (fig. 1). Except for the guide of neoan-tergan this activity is un-

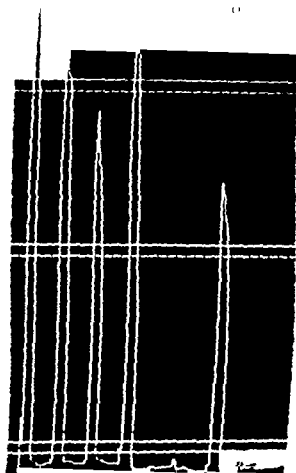


Fig. 1. Isolated guinea-pig's gut.

1: 0.010  $\gamma$  histamine. 2: 0.20 ml of Code-extract. 3: 0.008  $\gamma$  histamine. 4: Neoan-tergan in a concentration of  $10^{-7}$ .

distinguishable from that of histamine and may grossly mislead the observer if the test is performed only on the gut. The substance responsible for this non-histamine activity of the extracts was probably formed during the acid boiling, a possibility on which DALE quite recently (1948) has commented. In the present study gut-contracting activity is referred to as histamine only when it is entirely antagonized by adequate amounts of neoan-tergan.

The ultrafiltrates were prepared by a method originally described by REHBERG (1943) and adopted for histamine determination by EMMELIN (1945 a), who demonstrated that plasma histamine readily passes the cellophane membrane while substances which interfere with the assay on the guinea-pig's ileum are retained. In preliminary experiments EMMELIN (1945 b) found that ultrafiltrates of human plasma sometimes contained an agent which disturbs the assay on the gut. In my experiments this occurred only exceptionally. As a rule this method worked satisfactorily and gave figures which agreed fairly well with the histamine activity of the extracts.

From the *placentas* a cotyledone weighing about 5 gr was cut into pieces with scissors and ground in a mortar with quartz powder and trichloroacetic acid in order to arrest histaminolysis. The mixture was then extracted according to CODE. The chemical treatment of urine was carried out according to ANREP *et al.* (1944) involving the following steps: 1. The urine is shaken with charcoal, to which histamine is readily adsorbed. 2. Filtration, the filtrate being discarded. 3. The histamine is released from the charcoal through repeated washing with acidulated alcohol. 4. The alcoholic eluates are joined together and, after neutralisation, divided into two portions. 5. One of these portions is dried in vacuo on a boiling waterbath. The second is hydrolysed by boiling for 1.5 hours with HCl before evaporation to dryness. 6. The two fractions are then extracted repeatedly with alcohol and again evaporated to dryness. 7. The dry residues are taken up in water and neutralized with NaOH. 8. These extracts contain impurities, which may interfere with the final test. This material is removed by ad-

sorption to aluminium oxide. 9. After filtration and adjustment of the pH the extracts are ready for the final assay on the isolated guinea-pig's ileum. The histamine content of the non-hydrolysed sample corresponds to free histamine. The hydrolysed sample contains the total amount of histamine present. The difference between the two samples represents conjugated histamine. ANREP et al. did not assess the error of the method, but stated that added histamine was completely recovered. This is in agreement with my experience. It therefore seems justifiable to assume that the errors are of the same order as with plasma extracted according to CODE, i. e. about  $\pm 10$  p. c. (EMMELIN, KAHLSON and WICKSELL 1941).

*B. Methods for the determination of histaminolytic activity.* The histaminolytic activity of plasma was determined by a method described in a separate communication in this volume (WICKSELL 1949). The heparine used for prevention of clotting of the blood samples was in these experiments tricesolfree. The histaminolytic activity was expressed as  $\gamma$  of histamine base destroyed by one ml of plasma in one hour ( $\gamma$ /ml/h). The placenta, immediately after its delivery, was cleaned from superficial blood and weighed. A cotyledone, weighing about 5 gr, was minced and thoroughly ground in a mortar with quartz powder and four times its weight of Tyrode's solution. The mixture was allowed to stand for 24 hours at  $+4^{\circ}$  C. Observations by AHLMARK (1944) indicate that with this extraction procedure an approximate equilibrium will be established between the histaminolytic principle of the cellular and saline portions of the mixture. After centrifugation the supernatant fluid was removed from the mixture and its histaminolytic activity determined by a similar method as used for plasma. The histaminolytic activity is expressed as  $\gamma$  of histamine base inactivated by one gram placental tissue in one hour ( $\gamma$ /gr/h).

## Results.

### 1. The Histamine Content of Plasma and Urine in Parturition.

*A. Observations on plasma.* As mentioned above MARCOU et al. (1938) investigated the histamine content of total blood. If histamine should play a rôle in parturition as suggested by DALE and LAIDLAW (1910) and if this should manifest itself in a change in the blood histamine content it seems reasonable to assume that this change should take place in the plasma. I therefore preferred to study plasma instead of total blood, particularly since changes can occur in the histamine content of plasma without this being demonstrable in total blood. It is obvious that if histamine should be of interest in this connection it must be present in plasma in a physiologically active state; I therefore

Table 1.

*Histamine content of maternal plasma before, during and after delivery.*

Experiment Nr.	Before $\gamma/l$	During $\gamma/l$	After $\gamma/l$
1.....	< 10 (5)	< 10 (5)	< 10 (4)
2.....	< 10	< 10	< 10
3.....	17	15	18
4.....	< 10 (5)	—	< 10 (5)
5.....	37	34	26
6.....	< 10	< 10	< 10
7.....	—	< 10 (5)	< 10 (4)
8.....	< 10 (4)	—	< 10 (3)
9.....	< 10 (5)	< 10 (6)	

abstained from using the drastic chemical extraction procedure of CODE which might liberate active histamine from an inactive compound and instead used the less drastic ultrafiltration method.

For these experiments patients were selected, who were admitted to the clinic because of postmaturity or premature rupture of the bags of water, but who had no pains. If possible three samples of venous blood were collected from each patient, the first before the onset of pains, the second immediately before or a few minutes after the delivery of the foetus and the third during the confinement 1—3 days after parturition. The results of these experiments are summarized in table 1.

*B. Observations on urine.* ANREP et al. (1944) discovered that histamine is excreted in the urine not only in a free form, but also "conjugated" in an inactive form, from which the base can be released through hydrolysis. In my experiments I have determined both these fractions of histamine in the urine, using the technique of ANREP et al.

Urine was collected from the same group of patients as in the experiments on plasma. As with blood, the first sample was collected before the onset of pains and the third in the confinement. As far as possible both these samples were made up of the urine excreted in 24 hours. The second sample, which was obtained through catheterization consisted of the quantity contained in the urinary bladder at delivery. From each of the samples 10 ml were collected and used for the extraction. The results of the experiments on urine are given in table 2.

The observations confirm the statement of ANREP et al. that part of the histamine in human urine occurs in a conjugated form, from which it can be released through acid hydrolysis. The

Table 2.

*Histamine content of urine before, during and after parturition.*

Experiment Nr.	Time	Histamine in urine, $\gamma/l$		
		Total	Conjugated	Free
1	Before	65	20	45
	During	90	60	30
	After	240	145	95
2	Before	220	155	65
	During	180	140	40
	After	155	10	145
3	Before	200	175	25
	During	70	58	12
	After	55	33	22
4	Before	450	422	28
	During	680	540	140
	After	380	350	130
5	Before	3,400	3,190	210
	During	670	490	180
	After	90	25	65
6	Before	160	95	65
	During	135	95	40
	After	75	40	35
7	Before	300	255	45
	During	450	390	60
	After	300	255	45
8	Before	1,000	880	120
	During	—	—	—
	After	600	320	280
9	Before	—	—	—
	During	160	150	10
	After	180	80	100
10	Before	48	about 45	< 10
	During	—	—	—
	After	76	28	48
11	Before	50	—	—
	During	130	90	40
	After	< 10 (8)	< 10 (2)	< 10 (6)

figures of table 2 show great individual differences in the urinary excretion of histamine. Also in one and the same subject the figures



vary considerably in the different samples. This applies to free as well as conjugated histamine. These experiments are obviously open to some criticism; for instance samples 1 and 3 were collected over 24 hours whereas the urine of sample 2 was produced in the course of a relatively short period. It could be objected that a comparison of the histamine content of these samples is not justified as nothing is known about diurnal variations in the excretion of histamine. It seems however justified to conclude from these experiments, like those on plasma, that they lend no support to the hypothesis that histamine should play any part in delivery.

## 2. Histamine Content of Plasma in Relation to the Histaminolytic Activity.

*A. Histaminolytic activity of placenta and of maternal and foetal plasma.* In order to be able to assess the capacity of the placenta to destroy histamine I have carried out some incubation experiments on placental tissue. In these experiments I found it desirable also to determine the histaminolytic activity of maternal and foetal plasma. When the present study was begun no reports were published on the histaminolytic activity of foetal blood. Two years ago I started to carry out such determinations. In the meantime two papers on this problem came on record. ANREP, BARSOUM and IBRAHIM (1947), adopting their "Histamine-Index" method, reported that the histaminolytic activity of maternal serum is approximately three times stronger than of foetal serum. Quite recently SWANBERG (1948), employing AHLMARK's method, observed very low figures in foetal plasma; in his experiments the histaminolytic power of foetal plasma was as a rule less than 1/300 of maternal plasma.

In my experiments samples of placenta and of foetal blood were obtained at normal deliveries. Foetal blood was collected from the umbilical vein. The umbilical cord was clamped and cut immediately after the birth and before the pulsations in the cord had ceased. In order to prevent contamination with maternal blood or amniotic fluid the cord was carefully cleaned. The clamp was then released and the foetal blood remaining in the placenta was collected in a vessel containing heparine. Venous blood from the mother was withdrawn from a cubital vein within 15 minutes of delivery. Plasma was obtained by centrifugation and the

Table 3.

*Histaminolytic activity of placenta, maternal and foetal plasma.*

Experiment Nr.	Activity per gram placenta ( $\gamma$ /gr/h)	Total activity of placenta (mg hi/h)	Activity of maternal plasma ( $\gamma$ /ml/h)	Activity of foetal plasma ( $\gamma$ /ml/h)
1.....	—	—	5.0	0.021
2.....	42	25	3.0	0.011
3.....	220	99	8.6	0.018
4.....	66	44	4.6	0.009
5.....	88	48	3.8	0.009
6.....	130	74	4.0	0.007
7.....	75	53	4.0	0.023

histaminolytic activity of the samples estimated. Table 3 shows the results of these experiments.

In agreement with previous investigators I find that the histaminolytic activity of the placenta is very high. The whole organ is able to destroy between 25 and 100 mg of histamine in one hour. The foetal plasma has a very low activity of approximately the same order as observed in non-pregnant subjects. In my experiments the histaminolytic power of maternal plasma is 160—600 times stronger than of foetal plasma. My figures are of the same order as those of SWANBERG (1948).

Table 4.

*Histamine content of placenta.*

Experiment Nr.	Histamine content mg/kg
1.....	0.36
2.....	0.32
3.....	0.16
4.....	1.2
5.....	0.56
6.....	0.50
7.....	1.5
8.....	0.16
9.....	0.18
10.....	0.25

In this connection I have made some side-observations on the histamine content of placenta. The results of 10 experiments are given in table 4. It can be seen that the human placenta contains histamine in moderate amounts which demonstrates that histamine may be present in an organ concomitantly with strong

histaminolytic activity. This is in agreement with the original observations of BEST (1929) and BEST and McHENRY (1930) on other organs.

*B. Does the placental histaminase diminish the histamine content of foetal plasma?* Since the foetal blood has very weak histaminolytic activity it is obvious that histamine produced in the foetus only to a small extent can be inactivated in the foetal blood. On the other hand the placenta, owing to its high histaminolytic activity, seems well suited for the object of inactivating histamine. The following experiments were carried out to test this point. At normal human deliveries blood was collected in the following way. Immediately after delivery the foetal circulation was arrested through two clamps on the umbilical cord and the cord cut between the clamps. The clamp attached to the foetal stump of the cord was then released and about 15 ml of venous blood emerging from the foetal arteries collected in a vessel containing heparine. Finally the clamp on the placental stump was released and arterialised blood emerging from the placenta collected. Plasma was obtained and its histamine content determined. In most experiments the plasma volumes were too small to permit simultaneous determination with extracts and ultrafiltrates; in a few instances such duplicates were assayed.

The results of this series are given in table 5. The figures do not indicate that there is any destruction of histamine in foetal plasma flowing through the placenta. If there were any destruction of foetal histamine in the placenta this ought to manifest itself in differences in histamine content between arterial and venous foetal blood in view of the high histamine inactivating capacity of the placenta. From table 3 it can be seen that the placenta is able to destroy about 1,000  $\gamma$  in one minute. The foetal circulation through the placenta approximates 100—200 ml plasma per minute (BARCROFT 1946). This means that the placenta would be able to remove completely all the histamine present in the foetal plasma, even if the histamine concentration were as high as 5,000—10,000  $\gamma$ /l. It might be worth mentioning, however, that recent investigations indicate that most of the placental histaminase is present in the maternal parts of the placenta.

*C. Does the placental histaminase protect the foetus against histamine in maternal plasma?* It is known that tissues of old animals are considerably richer in histamine than those of young ones

Table 5.  
*Histamine content of foetal plasma ( $\gamma/l$ ).*

Experiment Nr.	Extracts		Ultrafiltrates	
	Arterial	Venous	Arterial	Venous
1.....	25	20	25	35
2.....	25	25	24	20
3.....	18	16	16	12
4.....	< 10 (8)	< 10 (9)	—	—
5.....	11	13	—	—
6.....	—	—	< 10 (8)	< 10 (8)
7.....	—	—	26	19
8.....	—	—	27	32
9.....	—	—	< 10 (8)	< 10 (7)
10.....	—	—	12	13
11.....	—	—	12	14
12.....	—	—	< 10 (7)	< 10 (7)
13.....	—	—	16	18
14.....	—	—	< 10 (4)	< 10 (5)
15.....	—	—	15	18
16.....	—	—	< 10 (3)	< 10 (2)
17.....	—	—	12	12

Table 6.  
*Histamine content of total blood of mother and foetus.*

Experiment Nr.	Mother $\gamma/l$	Foetus $\gamma/l$
1.....	92	120
2.....	76	120
3.....	77	400
4.....	50	85

(FELDBERG and KELLAWAY 1937). It might therefore be expected that maternal blood contains more histamine than foetal blood and if so it might be hypothesised that the function of the placental histaminase might be to protect the foetus against excess histamine. This assumption was tested in some preliminary experiments. A sample of arterial foetal blood was secured at the delivery as has been described earlier. Maternal venous blood was withdrawn at the same time. Table 6 gives a comparison between the histamine content of foetal and maternal total blood. Contrary to what might be expected foetal blood invariably contained more histamine than did maternal. In interpreting this observation it must be borne in mind that the proportion of cellu-

Table 7.

*Histamine content of maternal and foetal plasma.*

Experiment Nr.	Plasma			
	Ultrafiltrates $\gamma/l$		Extracts $\gamma/l$	
	Maternal	Foetal	Maternal	Foetal
1.....	—	—	12	14
2.....	—	—	20	17
3.....	—	—	28	35
4.....	—	—	< 15	< 15
5.....	—	—	12	13
6.....	—	—	43	40
7.....	—	—	73	67
8.....	—	—	28	28
9.....	—	—	45	35
10.....	—	—	18	25
11.....	—	—	35	35
12.....	17	14	19	16
13.....	20	27	—	—
14.....	< 10	< 10	—	—
15.....	< 10	< 10	—	—
16.....	32	30	—	—

lar elements is greater in foetal than in maternal blood and that the blood histamine is contained predominantly in the cells, particularly the leucocytes and platelets (SCHWARTZ 1936, CODE 1937 a and b, ZON, CEDER and CRIGLER 1939, MINARD 1941).

It was thought that better evidence might be obtained if plasma was used instead of total blood. Blood was collected in the same way as in the preceding series and the plasma was ultrafiltered or extracted. The results of these experiments are given in table 7. The findings give no support to the hypothesis that the placenta serves as a device to protect the foetus from the histamine present in the maternal blood. The fact that the histamine concentration in the plasma is approximately the same on both sides of the placental barrier indicates that it is carried by the circulation in a form permitting passage through cell membranes. This assumption is in agreement with the fact that similar histamine values are obtained with both the extraction and ultrafiltration methods.

This observation should also be correlated with EMMELIN's (1945 b) demonstration in cross circulation experiments that histamine is present in plasma in a physiologically active form. EMMELIN emphasized that though in an active state histamine

must not necessarily be present in a chemically free form. This was also suggested by DALE (1937—38). The experiments of table 7 indicate that at least part of the plasma histamine is present in such a form, that it can not be attacked by the histaminolytic principle present in plasma during pregnancy. In this connection it should, however, be pointed out that in many pregnancy plasmas investigated by me practically no histamine was found ( $< 10 \gamma/l$ ). Recently FRANDSEN (1948) claimed that a histaminase preparation (Torantil), which is known to be very impure and probably not very useful in this type of experiments failed to reduce the content of gut-contracting substances in the dog's serum. From this he concluded that histamine was not present in a free form. His experiments are in many respects open to criticism; in this connection I only want to recall the well known fact that serum contains gut-contracting substances other than histamine (ZIPF 1931, SIMON 1937, 1938, ZUCKER 1944, TSAI, Mc BRIDE and ZUCKER 1944).

In order to fortify the identity of the gut-contracting principle with histamine the specific antagonism of neoantergan was utilised in my experiments on plasma. In proper dosage this drug annulled all the activity here referred to as histamine. An experiment of this type is seen in fig. 2.

In agreement with EMMELIN (1945 b) I have observed that histamine occurs in maternal and foetal plasma in an ultrafilterable form and that this agent is unlikely to be present in plasma in a chemically free state, since it is not destroyed by the histaminolytic principle. There is a possibility, however, that plasma contains a fraction of chemically free histamine which is destroyed by the histaminase after withdrawal of the blood samples and before histaminolysis has been arrested. To test this possibility I have made use of the observation by ZELLER (1938) that hydroxylamine in a 0.01-molar concentration completely inhibits histaminolysis. Blood from women in late pregnancy was collected

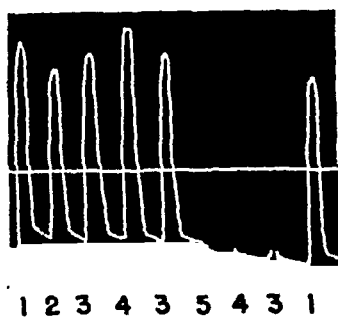


Fig. 2. Isolated guinea-pig's gut.

1: 0.014  $\gamma$  acetylcholine. 2: 0.008  $\gamma$  histamine. 3: 0.5 ml of the ultrafiltrate. 4: 0.010  $\gamma$  histamine. 5: Neoantergan in a concentration of  $10^{-7}$ .

Table 8.  
*Histamine content of plasma in pregnancy.*

Ex- peri- ment Nr.	Maternal plasma					Fœtal plasma  Histamine content γ/l
	No hydroxylamine		Hydroxylamine added			
	Histamino- lytic acti- vity γ/ml/h	Histamine content γ/l	Histamine content γ/l	Addition of histamine γ/l	Histamine content after addition of histamine γ/l	
1	0.80	< 10	< 10	10 20	17 36	—
2	1.2	< 10 (8)	< 10 (8)	100	78	—
3	1.3	< 10 (3)	< 10 (3)	50 100	45 85	—
4	3.3	< 10 (5)	< 10 (5)	200	170	—
5	1.0	< 10	< 10	200	160	—
6	5.3	90	80	500	500	90
7	3.8	100	110	500	550	100
8	—	—	40	—	—	43
9	—	—	22	—	—	25
10	—	—	87	—	—	93

in two vessels containing a few drops of heparine and one of them also a solution of hydroxylamine, which was calculated to give a final concentration of 0.01-molar in the sample. Plasma was obtained by centrifugation. In the plasma without hydroxylamine the histaminolytic activity was determined in one portion and another portion was incubated for one hour at 37° C and tested for its histamine content after extraction. The plasma containing hydroxylamine was also divided in separate portions, one of which was extracted without previous incubation. To other portions of this sample histamine in varying amounts was added. These samples were incubated for one hour and then extracted and tested for histamine. Special precautions to eliminate the added hydroxylamine were not necessary, because this substance was either destroyed during the extraction procedure or it did

not interfere with the assay on the guinea-pig's gut. Some of these experiments were carried out on plasma collected at delivery. In these cases foetal blood was also withdrawn and its plasma histamine content determined.

The results of these experiments are summarized in table 8. It can be seen from this table that the control experiments with added histamine show that the hydroxylamine had completely inhibited the activity of the histaminase. Small losses in added histamine are probably due to the extraction procedure. The table shows that, in spite of complete inhibition of the high histaminolytic activity, the histamine content is not higher in the hydroxylamine-treated samples than in the samples incubated without hydroxylamine. This finding gives no support to the assumption that a fraction of the histamine in maternal plasma occurs in a chemically free form. The observations suggest that in human pregnancy histamine is present in maternal and foetal plasma solely in an ultrafilterable, but not chemically free form.

No experiments described in this chapter support the hypothesis that the function of the histaminolytic principle in pregnancy is to protect mother or foetus against histamine.

### 3. Histamine Sensitivity in Pregnancy.

As a test for histamine sensitivity I have used the vascular reaction of the skin. The flare, one of the components of "the triple response" as described by LEWIS (1927), was considered to be well suited for this purpose. This reaction has the advantage of being locally restricted, excluding the risk of histamine absorption into the circulation in amounts sufficient to produce untoward effects. The size of this response was tested in 104 women. Of these 24 were non-pregnant, in fertile age, consulting because of gynaecological disorders such as sterility, secondary amenorrhoea or dysmenorrhoea. The other 78 were all healthy and in different stages of normal pregnancy. The age of pregnancy was calculated from the first day of the last menstruation, every month of pregnancy counting 28 days, *i. e.* delivery was expected at the end of the 10th month. In each of these subjects five intradermal injections were made into the skin of the upper arms or shoulder region. Histamine was injected in the following quantities: 0.01, 0.1, 1.0 and 10  $\gamma$ ; the fifth injection, serving as a control, was plain saline. The volume of each injection was 0.1 ml.



Table 9.

Patient Nr.	Preg- nancy month	Diameter of flare in cm for different histamine doses					Patient Nr.	Preg- nancy month	Diameter of flare in cm for different histamine doses			
		0.00	0.01γ	0.1γ	1.0γ	10.0γ			0.00	0.01γ	0.1γ	10.0γ
1	—	2.5	2	3	4	5	51	VII	1.5	1.5	4	6
2	—	2.5	2	3	4	5	52	VII	0	1.5	3	3
3	—	2.5	2	3	4	5	53	VII	1	2.5	4	5
4	—	3	3	4	4	5	54	VII	3	3	4.5	5.5
5	—	1.5	4.5	4	4.5	5.5	55	VII	2.5	2.5	4	5
6	—	0	2.5	4	4	5	56	VII	0	2.5	4	5
7	—	1.5	1	4.5	4	5	57	VII	1.5	3	4	5
8	—	0	2.5	4	4	5	58	VIII	4	3.5	5	6
9	—	2.5	4	4.5	4	5	59	VIII	1.5	4	4.5	5.5
10	—	2	1.5	3.5	4	5	60	VIII	4	3	5	6
11	—	0	2.5	4.5	4	5	61	VIII	0	0	4	5
12	—	2	1.5	3.5	4	5	62	VIII	0	0	4.5	5.5
13	—	0	2.5	4.5	4	5	63	VIII	0	0	0	4
14	—	1.5	4.5	4	4	5	64	VIII	0	1.5	3.5	4.5
15	—	2	0	3	5	6	65	VIII	0	2	4.5	5
16	—	0	1.5	4.5	4.5	5	66	VIII	0	0	3	4.5
17	—	4.5	3.5	4.5	5.5	6	67	VIII	0	2	3	4.5
18	—	0	2	4.5	5	6	68	IX	0	0	1	3
19	—	0	2.5	4.5	5.5	6	69	IX	0	0	1.5	3.5
20	—	1	0	3.5	4	5	70	IX	0	0	0.5	3.5
21	—	0	2.5	4	5	6	71	IX	0	0	4	5
22	—	1.5	5	5	5	6	72	IX	2.5	3	4	5
23	—	2	3.5	5	5	6	73	IX	0	0	1.5	4.5
24	—	2	3.5	5	5	6	74	IX	2.5	3	4.5	5.5
25	II	2	3.5	5	5	6	75	IX	0	2	2	4
26	III	1	4	4.5	5	6	76	IX	0	1	1	4
27	III	2	4	5	5.5	6	77	IX	1	0	2.5	4.5
28	III	1	2	3	4	5	78	IX	1	1	3	4
29	III	2	2.5	3.5	4	5	79	IX	0	0	4.5	5.5
30	III	2	3	4	5	6	80	IX	0	0	4	5
31	III	0	4	4.5	5	6	81	IX	0	0	3	4
32	III	0	3.5	4	5	6	82	IX	0	0	3	4
33	IV	3	3.5	4.5	5	6	83	IX	0	0	3	4
34	IV	2	3	4	5	6	84	IX	0	0	3	4
35	IV	2	3	4	5	6	85	IX	0	0	3	4
36	IV	0	4	4.5	5	6	86	IX	0	0	3	4
37	V	0	4	4.5	5	6	87	IX	0	0	3	4
38	V	0	1.5	3	4	5	88	IX	0	0	3	4
39	V	0	2	3	4	5	89	IX	0	0	3	4
40	V	2	2	3	4	5	90	IX	0	0	3	4
41	V	2	2	3	4	5	91	X	0	0	3	4
42	VI	2	2	3	4	5	92	X	0	0	3	4
43	VI	0	1.5	3	4	5	93	X	1.5	2	3	4
44	VI	0	2	3	4	5	94	X	0	0	3	4
45	VI	0	2	3	4	5	95	X	0	0	3	4
46	VI	2.5	2	3	4	5	96	X	0	0	3	4
47	VI	1	2	3	4	5	97	X	0	0	3	4
48	VI	1	2	3	4	5	98	X	0	0	3	4
49	VII	1	2	3	4	5	99	X	1.5	2	3	4
50	VII	0	2	3	4	5	100	XI	1.5	2	3	4
							101	XI	2	3	4	5
							102	XI	2	3	4	5

Table 10.

*Response to intradermal histamine injections in pregnant and non-pregnant women.*

Pregnancy month	Number of observations	Diameter of flare in cm for different histamine doses				
		0.00	0.01 $\gamma$	0.1 $\gamma$	1.0 $\gamma$	10.0 $\gamma$
Controls .....	24	1.5	3.2	4.2	5.2	6.3
II .....	1	2	3.5	5	6	7
III .....	7	2.1	3.4	4.6	5.6	6.4
IV .....	4	1.8	3.5	4.8	5.6	6.6
V .....	5	0.3	2.6	4.2	5.1	6.1
VI .....	7	1.2	2.1	3.7	4.6	5.6
VII .....	9	1.2	2.5	3.9	4.7	5.7
VIII .....	10	1.2	2.0	4.0	4.6	5.6
IX .....	23	0.6	2.3	4.0	4.7	6.0
X .....	9	0.9	2.1	3.9	4.9	6.1
XI .....	3	1.7	2.5	5.0	5.2	5.7

Controls .....	24	1.5	3.2	4.2	5.2	6.3
II—VI .....	24	1.4	2.9	4.3	5.2	6.2
VII—XI .....	54	0.9	2.3	4.0	4.7	5.9

The response was inspected in bright daylight when the flares were at their height, which usually occurred after 3—5 minutes. The largest and the smallest diameter of each flare was measured in cm and the mean value taken.

The results of these observations are given in table 9 and summarized in table 10. As can be seen from the tables these experiments give no indication that the sensitivity of the skin vessels to injected histamine is altered in pregnancy.

### Discussion.

The remarkable fact that the histaminolytic activity is strongly increased in pregnant women must give rise to the question whether histamine is in some way engaged in normal or pathological processes in pregnancy. Unfortunately experiments on this problem are rendered difficult by the fact that the increase in histaminolytic activity is considerable only in human beings. In the present investigation the following attempts were made to find a connection between histamine and pregnancy. A comparison was made between the histamine content of plasma, before, dur-

ing and after delivery. This period of pregnancy was chosen for two reasons, firstly because histamine is known to stimulate the motor activity of the uterine muscle and secondly because the histaminolytic activity reaches a maximum about delivery and then rapidly declines. In this connection samples of urine were also examined for their content of free and conjugated histamine as the urinary excretion of histamine seems to be changed in some way in pregnancy. In a second series of experiments my interest was directed on the fact that the placenta has a very high histaminolytic power and an attempt was made to find out if this activity gives rise to any differences in the histamine content of maternal and foetal plasma or between arterial and venous foetal plasma. In the third group of experiments the sensitivity to injected histamine was examined in pregnant and non-pregnant women. STRAUSS and CASTLE (1932) found that the ability of histamine to stimulate gastric secretion is diminished in pregnancy. WAY (1945) found this decrease to be inversely related to gonadotropic activity. It seems improbable that this decrease in response to histamine can be correlated with the histaminolytic activity as the effect of histamine on gastric secretion according to STRAUSS and CASTLE again became normal towards term; in this period the histaminolytic activity is still high. In my experiments I have used the response of the small blood vessels of the human skin as a test for histamine sensitivity. The experiments of the present paper do not give any evidence that histamine plays a rôle or that the histamine metabolism is in any way affected in normal human pregnancy. Nothing is known as to the significance of the high histaminolytic power in pregnancy. Possibly it might prove more fruitful to see if there is any connection between histamine and pregnancy under pathological conditions, for instance toxæmias of pregnancy, some symptoms of which could be due to histamine. In this connection it is also of interest to note that the pregnancy toxæmias like the very high increase in histaminolytic power occur only in human beings. In many cases of toxæmia the histaminolytic activity is found to be abnormal (AHLMARK 1944).

### Summary.

1. No changes were found to take place in the histamine content of plasma or urine during parturition. In agreement with

earlier investigators urine was found to contain histamine in a free form and also in a conjugated form, from which it can be liberated through acid hydrolysis.

2. The histaminolytic activity of placenta is very high. In spite of this histamine is present in this organ. Contrary to what is the case in maternal plasma the histaminolytic power of foetal plasma is low. No decrease in the histamine content takes place when foetal plasma flows through the placenta. The histamine content of maternal and foetal plasma is of the same order. Histamine seems to be present in the plasma in a form which is ultrafilterable but not attacked by the histaminase.

3. The size of the flare of the triple response caused by intra-dermal injections of histamine is of the same order in pregnant and non-pregnant women.

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From the Department of Physiology, University of Lund.

## The Histamine Sensitivity of Smooth Muscles in Pregnant Guinea-Pigs.

By

FINN WICKSELL.<sup>1</sup>

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The experiments of this paper were carried out in order to test if the high histaminolytic activity of plasma in pregnancy is accompanied by a decreased histamine sensitivity. As is well known histamine exerts its main effects on blood vessels, smooth muscles and the secretory cells of the gastric mucosa. In a previous paper in this volume (WICKSELL 1949) I have examined the responses to histamine of the small blood vessels in the skin of pregnant and non-pregnant women. In that paper some experiments on gastric secretion provoked by histamine in human pregnancy were also quoted. Experiments on the responses of smooth muscles to injected histamine might involve a certain risk in pregnancy; DALE and LAIDLAW (1910) for instance found that after injection of histamine into pregnant cats the foetuses were expelled dead. My experiments on smooth muscles, which are described in the present paper, were therefore carried out on guinea-pigs. It should be borne in mind that the histaminolytic activity in pregnancy is particularly pronounced in man; but also in guinea-pigs a definite increase takes place (AHLMARK 1944). These animals are well suited for experiments of this type also because of the fact that their smooth muscles are very sensitive to histamine. My first experiments were carried out on the bronchi, but I soon found it desirable also to use some other smooth muscle prepara-

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<sup>1</sup> This paper forms part of a thesis presented to the Medical Faculty, University of Lund, for obtaining the degree of M. D.

tion. The urinary bladder of the guinea-pig, which to my knowledge has not been used in earlier studies on histamine, was found to be a convenient *in vivo* test object.

### Methods.

The experiments were carried out in chloralose anaesthesia. The blood pressure, which in guinea-pigs is comparatively insensitive to histamine, was recorded from the carotid artery by a membrane manometer and served as a control of the general condition of the animals. The bronchial tone was registered by a method of KONZETT and RÖSSLER (1940) as used in this laboratory (EMMELIN, KAHLSON and WICKSELL 1941). Injections were made into a jugular vein.

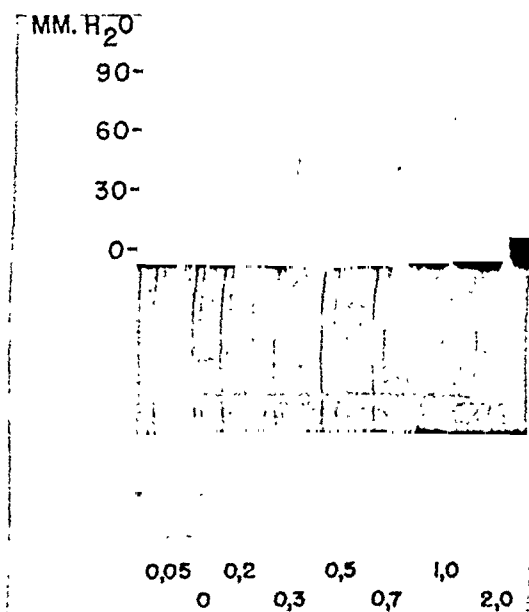


Fig. 1. Pregnant guinea-pig under chloralose. Registration from above: Motor activity of the urinary bladder (bromophorm manometer), bronchial tone, time in minutes and signal.

*Experiments on the urinary bladder.* An incision was made in the lower part of the abdomen and the urethra between the symphysis and the bladder was dissected and opened. A cannula was inserted through the urethra into the bladder and connected with a U-shaped manometer, filled with water or bromophorm. The bladder was filled with 3–5 ml saline giving an initial pressure of 5–10 cm  $H_2O$ . The sensitivity of the bladder to histamine is demonstrated in fig. 1. The intravenous injection of 0.05  $\gamma$  histamine (base) was followed by an increased motor activity of the bladder; 0.7  $\gamma$  of histamine was neces-

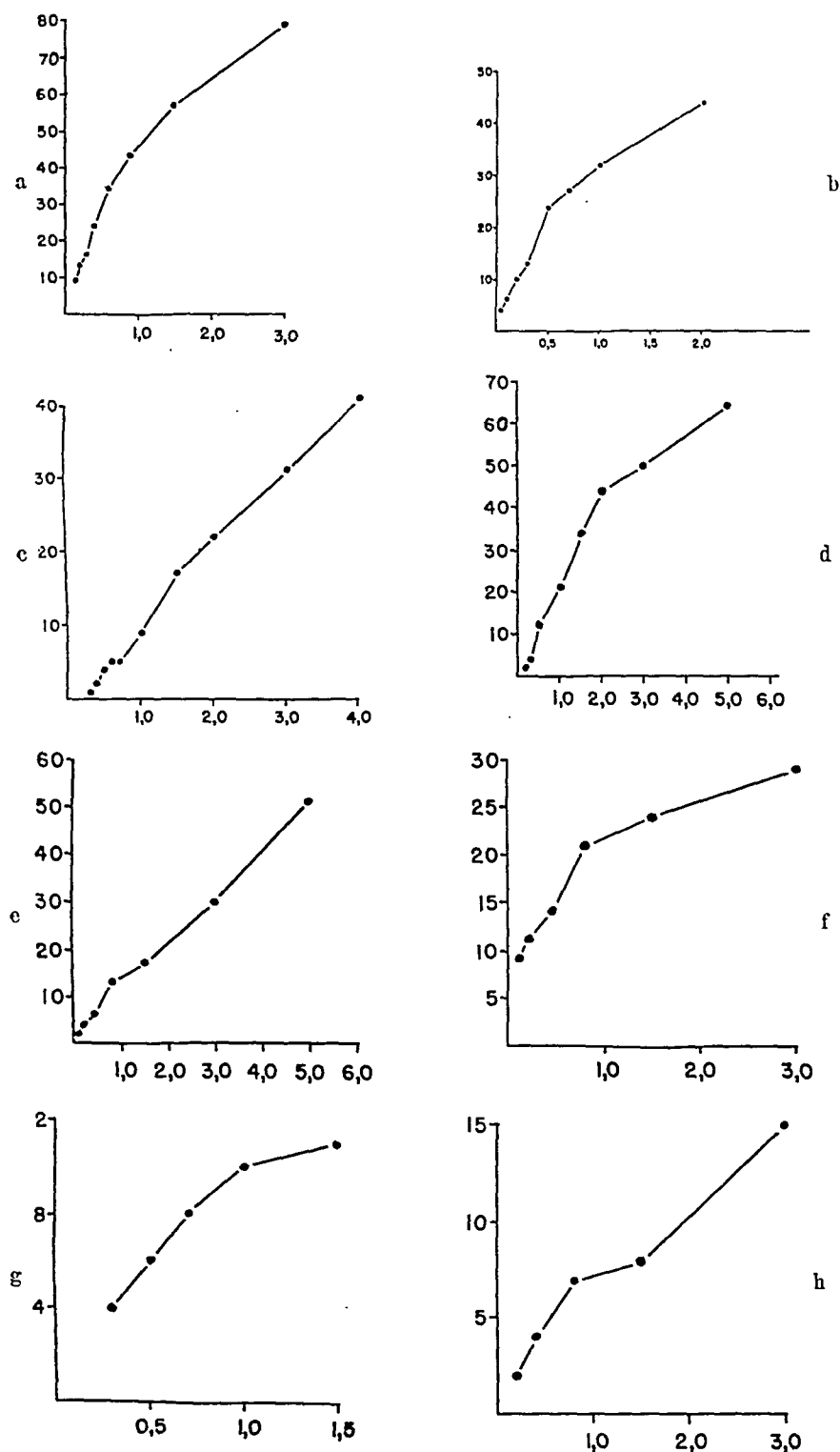


Fig. 2. Dose-response curves of the motor activity of the urinary bladder of guinea-pigs after intravenous injections of histamine.

Abscissa: Doses of histamine injected (base in  $\gamma$ ).

Ordinate: Increase of pressure in the bladder (mm H<sub>2</sub>O).



sary to cause bronchoconstriction in this experiment. In 12 experiments on non-pregnant animals the lower limit of sensitivity of the urinary bladder had a mean value of  $0.35 \gamma$  histamine/kg body weight (ranging from  $0.05$ — $0.85 \gamma/\text{kg}$ ). The corresponding figures for the bronchi were  $0.80 \gamma/\text{kg}$  (ranging from  $0.35$ — $2.4$ ). Generally the urinary bladder was found to be twice as sensitive as the bronchi. Fig. 2 shows dose-response curves of the urinary bladder from 8 experiments. With

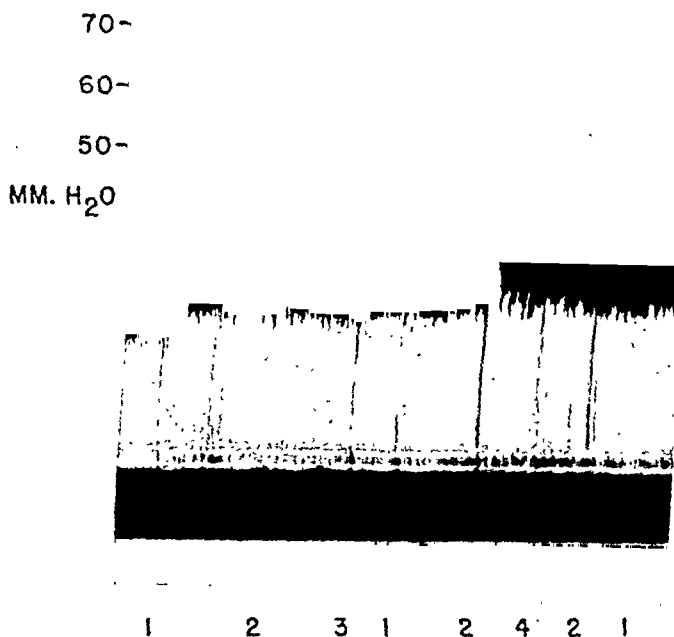


Fig. 3. Male guinea-pig under chloralose. Registration from above: Motor activity of the urinary bladder (water manometer), bronchial tone, time in minutes and signal.

this method I found it possible to discriminate between doses of histamine differing with more than 50 p. c.

In order to test the *specificity* of the bladder preparation to histamine its sensitivity to acetylcholine was also examined. An experiment of this type is shown in fig. 3. In these experiments  $10$ — $20 \gamma$  of acetylcholine, given intravenously, usually elicited a moderate contraction of the bronchi, but no responses were obtained from the bladder. As much as  $100$ — $200 \gamma$  of acetylcholine, which strongly affected the blood pressure, had to be given in order to elicit a response from the urinary bladder. On close arterial injection into a renal artery, on the other hand, the bladder responded to doses of acetylcholine as small as a few  $\gamma$ , the discrepancy in dosage not being surprising considering the rapid destruction of this substance by cholinesterase.

Table 1.

*Histamine sensitivity in male guinea-pigs.*

Experiment Nr.	Weight of animal in grams	Urinary bladder doses in $\gamma$		Bronchi doses in $\gamma$	
		Biggest dose found inactive	Smallest dose found active	Biggest dose found inactive	Smallest dose found active
1 .....	780	—	—	0.70	1.00
2 .....	580	—	—	0.50	1.00
3 .....	640	—	—	0.50	1.00
4 .....	600	—	—	0.05	0.15
5 .....	620	—	—	0.10	0.20
6 .....	560	—	—	0.30	0.50
7 .....	390	—	—	0.50	1.50
8 .....	500	—	—	0.20	0.30
9 .....	430	—	—	1.00	1.50
10 .....	410	—	—	0.01	0.03
11 .....	420	0.10	0.30	0.30	0.40
12 .....	430	0.10	0.30	0.20	0.30
13 .....	440	0.20	0.40	0.20	0.40

Table 2.

*Histamine sensitivity in non-pregnant female guinea-pigs.*

Experiment Nr.	Weight of animal in grams	Urinary bladder doses in $\gamma$		Bronchi doses in $\gamma$	
		Biggest dose found inactive	Smallest dose found active	Biggest dose found inactive	Smallest dose found active
14 .....	740	—	—	1.00	2.00
15 .....	800	—	—	0.50	1.00
16 .....	740	—	—	0.50	0.80
17 .....	380	—	—	0.10	0.20
18 .....	330	—	—	0.70	1.00
19 .....	410	—	—	0.07	0.10
20 .....	800	0.30	0.40	0.30	0.40
21 .....	840	0.30	0.50	0.10	0.20
22 .....	800	0.05	0.10	0.20	0.30
23 .....	750	0.05	0.10	0.30	0.40
24 .....	690	0.10	0.15	0.30	0.60

*Neonantergan* in doses of 5—20  $\gamma$  rendered the urinary bladder as well as the bronchi completely insensitive to histamine in the small doses employed in my experiments (Fig. 3). *Atropine* in doses which made the bronchi insensitive to acetylcholine did not significantly alter the sensitivity to histamine.

## Results.

In 37 guinea-pigs the sensitivity of the bronchi and the bladder to histamine was examined, this group consisting of 13 males, 11 virginal females and 13 pregnant females. The smallest dose of histamine, which produced definite effects on the bronchi and the bladder respectively, was determined. The results of the experiments on males are given in table 1, those on virginal females in table 2 and on pregnant females in table 3. The data of these three tables are summarized in table 4.

Table 3.  
*Histamine sensitivity in pregnant guinea-pigs.*

Experiment Nr.	Weight of animal in grams	Length of foe- tuses in cm.	Urinary bladder doses in γ		Bronchi doses in γ	
			Biggest dose found inactive	Smallest dose found active	Biggest dose found inactive	Smallest dose found active
25 .....	770	2.5	0.10	0.20	0.70	1.00
26 .....	690	2.5	0.30	0.30	0.20	0.30
27 .....	1 000	5	—	—	5.00	10.00
28 .....	820	7	0.10	0.20	0.20	0.30
29 .....	740	9	0.30	0.40	0.60	0.80
30 .....	780	9	0.30	0.50	2.00	3.00
31 .....	1 140	10	—	—	2.50	4.00
32 .....	750	10	0.00	0.05	0.50	0.70
33 .....	690	12	0.15	0.20	1.50	2.00
34 .....	840	14	0.20	0.30	0.70	1.00
35 .....	770	15	0.20	0.30	3.00	5.00
36 .....	800	15	0.10	0.20	3.00	5.00
37 .....	920	16	0.05	0.10	7.50	10.00

Table 4.  
*Mean values on histamine sensitivity in the different groups.*

Group	Weight in grams	Urinary bladder doses in γ		Bronchi doses in γ	
		Smallest active dose per animal	Smallest active dose per kg	Smallest active dose per animal	Smallest active dose per kg
Males .....	520	0.33	0.63	0.64	1.21
Non-pregnant fe- males .....	660	0.25	0.38	0.64	0.96
Pregnants .....	820	0.25	0.30	3.32	4.03

As to the bronchi the histamine sensitivity seems to be approximately the same in males and virginal females, whereas the sensitivity in pregnancy seems to be markedly decreased. The sensitivity of the urinary bladder, on the other hand, is not significantly altered in pregnancy.

### Comment.

The results from the experiments on bronchi seem to suggest that the sensitivity of smooth muscles to histamine is decreased in pregnancy; but in evaluating these results it has to be borne in mind that anaesthetics like chloralose and urethane greatly decrease the sensitivity of certain smooth muscles to histamine (FARMER 1937, 1938), and it is well known that for anaesthesia bigger doses of narcotics are required in pregnant than in non-pregnant animals. In fact, in my experiments it was usually necessary in pregnant animals, anaesthetized with chloralose in the usual way, to add urethane in an amount which alone should have given satisfactory anaesthesia in a non-pregnant animal. From table 3 can be seen that the more advanced the pregnancy is the lower is the histamine sensitivity of the bronchi. This gradual decrease seems to have no relation to the histaminolytic activity of plasma for it is known that the histaminolytic power reaches its maximum about the middle of the gestation period, to diminish again towards term. In view of these facts the results from the experiments on bronchi and bladder can scarcely be said to indicate that there is any decrease in histamine sensitivity of smooth muscles in pregnancy, which can be correlated to histaminolytic activity. It may be added that the results from experiments on human skin in pregnancy pointed in the same direction (WICKSELL 1949).

### Summary.

1. A method for using the guinea-pig's urinary bladder as an *in vivo* test for histamine is described.
2. The sensitivity to histamine of the bronchi and the urinary bladder is tested in male, virginal female and pregnant guinea-pigs. These experiments do not indicate that there is any decrease in histamine sensitivity of smooth muscles in pregnancy which can be correlated with the increased histaminolytic activity of plasma.

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From the Department of Physiology, University of Lund.

## The Action of Corticotropic and Adrenal Cortex Hormones on the Mammary Gland.

By

DORA JACOBSON.

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In a recent study on parabiotic rats JACOBSON (1948) observed that mammary gland growth occurs in the hyp.ect. co-twin of a partner with intact hypophysis when the latter is injected with high doses of estrogens. However, administration of estrogens into the hyp.ect. co-twin of a partner with intact hypophysis does not elicit mammary gland growth in the hyp.ect. parabiont. From the evidence put forward JACOBSON concluded that estrogens are capable of releasing an anterior hypophyseal factor with mammogenic properties. In the paper just mentioned no attempt was made to elucidate the nature of the postulated factor.

It is well established that the anterior pituitary gland plays an important rôle in the normal process of mammary gland development. However, in spite of a large amount of work (PETERSEN, 1944, FOLLEY, 1947) it is still open to discussion, to which hormones the mammogenic action of the hypophysis can be ascribed. Amongst the known hormones of the anterior pituitary gland the effect exerted by the corticotropic factor has been studied by only a few investigators and the results are conflicting (NELSON, 1941, 1943, COWIE and FOLLEY, 1947). The investigations of CHRISTENSEN (1944), PINTO (1945 a, b) and KIMELDORF and SODERWALL (1947), suggest that estrogens are capable of releasing the adrenotropic factor from the anterior pituitary gland.

In the present paper the relationship between the reactions of the mammary glands and the adrenals is studied on parabiotic

rats. This technique and its suitability for investigations of this kind is discussed in a previous paper (JACOBSON, 1948).

*Experimental:* The animals are virgin female albino rats from an inbred strain kept at our department and fed ad libitum on table scraps, milk and grain.

The parabiosis (coelio-anastomosis) is made according to the method described in my previous paper (1948). The experiments shown in table 1 and 2 are done on parabiotic pairs used for the foregoing study. The age of all animals is about 21 days at parabiosis, and 3 to 5 months at autopsy. The present investigation includes 57 pairs of parabiotic rats.

For the different types of experiments performed 1) both members of the parabiosis are castrated, and one of them is hypophysectomized, 2) one partner is castrated, and the other one is adrenalectomized, and 3) the ovaries and adrenal glands are removed from one parabiont, the co-twin is hypophysectomized.

The hyp.ect. is a slight modification of the method described by COLLIP, SELYE and THOMSON (1933). The hypophysis as well as the adrenals are in all cases removed after established parabiosis. The entire removal of the gland is controlled by means of microscopic examinations of serial sections throughout the hypophysis capsule and adjacent tissues. The absence of the adrenals is checked macroscopically, and in doubtful cases microscopically.

The preparations used for estrogen and progesterone treatment are Di Menformon (estradiol monobenzoate in olive oil) and crystalline progesterone.<sup>1</sup> Di Menformon is given intramuscularly every other day in doses of 10 and 100  $\mu$ g (0.01 ml of a solution of 10,000 i. u. per ml, and 0.02 ml of a solution of 50,000 i. u. per ml) using a 0.5 ml tuberculin syringe with a coloured plunger. A suspension of 5 mg cryst. progesterone in about 2 ml 0.9 per cent saline solution is injected subcutaneously every other day.

The mammary glands are studied on whole mount preparations stained with gallocyenin chromalum (EINARSON, 1932, LAGERSTEDT, 1947), and on 5 to 10  $\mu$  thick paraffin sections stained with hematoxylin eosin. Details about the technique are given in my previous paper. The material presented includes 130 mammary glands, examined according to the principles already described (COWIE and FOLLEY, 1947, JACOBSON, 1948). In the present paper growth and development of the mammary gland is denoted by "+" where the number of ducts and side buds is obviously increased, and/or acini and secretion are present. The observations made on the mammary glands are illustrated by figures and will be described when presenting the experiments.

The adrenal glands are removed at autopsy, fixed in 10 % formalin and weighed on a torsion balance (1 interval = 1 mg). Frozen sections, 5 to 10  $\mu$  thick, stained with colloidal sudan are examined microscopically. An atrophy indicated by a reduction of the cortex and a loss of

<sup>1</sup> Di Menformon was kindly supplied by the Pharmacia Ltd., Stockholm, Progesterone by the Nordiska Organon Ltd., Stockholm.

sudanophilic granules in the fasciculate and the reticulate zones (SMITH, 1930) is qualified as "sudanophobic zone" (REISS *et al.*, 1936) (+), +, ++, +++.

Four groups of experiments will be reported. They are presented and commented on separately before discussing the results of this investigation.

## Results.

### Group A.

*A non hyp.ect. castrated rat is joined in parabiosis with a hyp.ect. rat. The hyp.ect. partner is injected with estradiol benzoate and progesterone (fig. 6, I). The experiments are summarized in table 1.*

Table 1 shows 15 experiments. In 11 cases the hypophysis is completely or practically completely removed. In expts. 12 to 15 a more or less large remnant of the anterior pituitary gland has been left after hyp.ect. With the exception of expts. 1, 6, 8 and 12 the hyp.ect. parabiont has been treated with estrogens and progesterone according to the following scheme: 1) 10  $\mu$ g and later 100  $\mu$ g estradiol benzoate every other day, each dose continued for about a fortnight, and 2) for about a fortnight 100  $\mu$ g estradiol benzoate every other day, and on the alternate day 5 mg progesterone. In expt. 1 and 6 the hyp.ect. parabiont was not treated with estradiol benzoate alone. In expt. 8 and 12 the hyp.ect. co-twin of the castrated mate was not castrated. Injections of 5 mg crystalline progesterone were given alone. The ovaries presented an excessive hypertrophy. Details about the treatments of the animals as well as about the reaction of the mammary glands are given in my previous paper (tables 6 to 9 a), and the results are discussed extensively as far as the mammary glands are concerned. Therefore it is only to be stated now that the mammary glands of the parabiotic twins remain undeveloped inspite of large amounts of ovarian hormones circulating in the hyp.ect. partner. When the hypophysis has not been removed entirely (expts. 12—15), mammary gland growth does, however, occur.

The adrenal glands are examined at different intervals from 43 to 55 days after the first injection of either of the ovarian hormones. An atrophy characterized by reduced weight (mean for both glands =  $10.2 \pm 0.05$ ) and a complete loss of sudanophilic granules in the fasciculate and reticulate zones is present in



the adrenals of the hyp.ect. parabionts, even in those with a remnant of anterior pituitary lobe and hypertrophic mammary glands.

#### *Group A: Comments.*

In 1935 DU SHANE *et al.* observed that the atrophy of the adrenal glands occurring in single rats after hyp.ect. is not restored when the hyp.ect. rat is united in parabiosis with a partner with an intact hypophysis. Observations of my own (unpublished) on twins consisting of a castrated and a hyp.ect. rat confirm those of DU SHANE *et al.* My experiments showed further that in spite of the development of hypertrophic ovaries mammary gland growth does not occur in the hyp.ect. co-twin of a castrated rat (JACOBSON, 1948).

The expts. of table 1 show that the atrophy of the adrenal glands is not repaired by administering high doses of estradiol benzoate and progesterone into the hyp.ect. co-twin of a partner with intact hypophysis. Since it is established that even large quantities of ovarian hormones circulating in one parabiont do not exert an action on the hypophysis of the co-twin, the result of the expts. of group A support the view that the stimulating effect of the ovarian hormones on the adrenal glands is mediated by the hypophysis (CHRISTENSEN, 1944). The observation that mammary gland growth does occur in incompletely hyp.ect. parabionts in spite of atrophic adrenals suggests that neither the corticotropic hormone nor the adrenal cortex are of major importance for the development of the mammary gland. The importance of the hypophysis is confirmed, however.

In the following group B the reaction of the adrenal glands is investigated after modifying the experiments so as to obtain mammary gland growth in the hyp.ect. co-twin of a partner with intact hypophysis.

#### *Group B.*

*A non hyp.ect. castrated rat is joined in parabiosis with a hyp.ect. castrated rat. The non hyp.ect. partner is injected with estradiol benzoate and later progesterone is given to the hyp.ect. co-twin (fig. 6, II). The experiments are summarized in table 2.*

Table 2 includes 14 experiments. The doses and the periods of the injections of estradiol benzoate and progesterone are the same as those used for the preceding group A. But in the present

group B all injections of estradiol benzoate are given to the partner with intact hypophysis. Since details about the treatment as well as about the mammary glands are given in my previous paper (especially in table 13), it is only briefly mentioned that an extensive mammary gland growth was promoted in all the parabiotic twins examined.

The adrenal glands are studied concomitantly with the mammary glands at different intervals from 34 to 70 days after the first injection of estradiol benzoate into the non hyp.ect. partner. A reduction of the weight and a development of a broad sudanophobic zone (fig. 1) are observed in the adrenal glands of all the hyp.ect. parabionts. (Mean weight of both adrenals of the hyp.ect. partners =  $10.3 \pm 0.08$  mg.)

#### *Group B: Comments.*

Two observations made on the experiments of table 2 are emphasized: 1) The adrenal glands of the hyp.ect. parabiont remain atrophic in spite of the administration of high doses of estrogens into the co-twin with intact hypophysis, and 2) simultaneously with the atrophic adrenals hypertrophic mammary glands are present.

1) This observation is not opposed to the view, mentioned before, that estrogens can release the corticotrophic hormone from the anterior lobe of the pituitary gland. However, it shows that the corticotrophin does not circulate in the parabiont with intact hypophysis in quantities sufficiently increased to exert an effect upon receptor organs in the hyp.ect. co-twin.

2) This result suggests that the hypophyseal factor claimed by JACOBSON (1948) to be released by the action of estrogens (p. 423) is not corticotrophin, and that the adrenal cortex hormones do not play an important rôle in the process of mammary gland development.

In the following groups C and D the problem is attacked from another angle.

#### *Group C.*

*A castrated rat is joined in parabiosis with an adrenalectomized rat* (fig. 6, III). The experiments are summarized in table 3.

Table 3 summarizes 19 observations made on the mammary glands of 11 parabiotic twins. In nos. 63, 66, 67 and 68 adrenalectomy is performed 18 to 20 days after parabiosis and castration

of the co-twin. In these experiments the ovaries and mammary glands of the non-castrated parabiont are hypertrophic when the adrenals are removed. In nos. 174—180 castration of one partner is performed simultaneously with adrenalectomy of the other one. Here neither ovaries nor mammary glands hypertrophied prior to the removal of the adrenal glands. The examination of ovaries and mammary glands, made at different intervals from 14 to 72 days after adrenalectomy reveals a uniform result: The ovaries are hypertrophic, containing numerous cystic follicles and in most cases well developed corpora lutea. The mammary glands of the adrenalectomized animal show numerous, evenly distributed side buds and acini, often with secreting cells. The picture of the whole mount preparation as well as that of the paraffin section is similar to that of a normal female united with a castrated one (ZECKWER, 1944, JACOBSON, 1948). The mammary glands of the castrated parabionts remain undeveloped.

#### *Group C: Comments.*

The result of the experiments of table 3 (fig. 3) shows that even in the absence of the adrenals mammary gland growth occurs in the co-twin of a castrated mate. Thus, there is a striking difference between the effect of an adrenalectomy compared with that of a hyp.ect., which, as mentioned before (p. 426), makes the mammary gland unresponsive to the stimuli exerted by the hormones of the hypertrophic ovaries. Before discussing these observations as to their bearing on the relationship between the adrenal cortex and the mammary gland the following question has to be dealt with: is it likely that adrenal cortex hormones are carried from the intact parabiont into the adrenalectomized co-twin? The lifetime of a single adrenalectomized rat is very short (max. 10 days). The adrenalectomized co-twin of a partner with intact adrenals possesses according to my experience a low resistance to infection and inanition. But, provided that the animals are thoroughly cared for, they can be maintained in perfect health for several months (tables 3 and 4). Regarding the conditions regulating the exchange of metabolites between parabiotic twins (discussed in my previous paper) it is unlikely that the hormones secreted by the adrenal glands of one parabiont cross over into the adrenalectomized co-twin. Disturbances of the carbohydrate metabolism and electrolyte balance such as those occurring after adrenalectomy can, however, be repaired through

the action of the intact organism of the co-twin. Since it has been shown that single adrenalectomized animals can be maintained in good basal condition by administering sodium salts, it seems reasonable to assume that normalization of the electrolyte and carbohydrate balance constitutes the factors responsible for the prolongation of the lifetime of the adrenalectomized co-twin of a normal partner. The result of the experiments of group C points to an independence of mammary gland growth from the adrenal glands.

In the following group D the experiments are modified so as to allow the study of a hyp.ect. animal with non atrophic adrenals.

#### *Group D.*

*A castrated adrenalectomized rat is joined in parabiosis with a hyp.ect. rat (fig. 6, IV). The experiments are summarized in table 4.*

Table 4 summarizes observations made on 17 parabiotic twins. In expts. 1—10 the hyp.ect. is complete, and in expts. 11—17 a remnant of the anterior lobe has been left after hyp.ect. The adrenalectomy is performed a fortnight to three weeks after parabiosis. With the exception of expt. 4 the adrenalectomized partner is castrated about 4 weeks after parabiosis, simultaneously with the hyp.ect. of the co-twin. In expt. 4 castration was done at parabiosis, a fortnight prior to adrenalectomy, and 30 days preceding the hyp.ect. of the co-twin. The mammary glands, ovaries and adrenals are examined at different intervals from 25 to 62 days after hyp.ect.

The ovaries are hypertrophic, their weight greatly exceeds that of normal adult rats in all but 3 cases (expts. 1, 3, 12). Cystic follicles and well developed corpora lutea are present. The weight of the adrenals is two to four times that of a hyp.ect. partner of a non adrenalectomized co-twin (mean for both glands =  $35 \pm 2.5$  mg), and the same as that found in group C in the parabiont with intact hypophysis (mean for both glands =  $37 \pm 2.4$  mg). In all but one of the completely hyp.ect. parabionts (expt. 2), and in all but 3 of the incompletely hyp.ect. partners (expts. 14, 15, 17) the size and the lipid content of the adrenal cortex are like those of the adrenals of normal rats (fig. 2). In the expts. 2, 14, 15 and 17 a more or less well defined sudanophobic zone is present.

The mammary glands of all the completely hyp.ect. parabionts reveal roughly uniform changes. The whole mount preparation shows a compact mass of thick ducts and side buds, often only

in the central part of the gland, while the periphery is made up by a sparse system of bare thin ducts. The contrast between the compact center and the atrophic periphery in these glands is striking (fig. 4). The area covered by the gland is not enlarged. The microscopic examination of sections reveals an atypical growth of the cells covering the walls of large ducts. The size as well as the number of these epithelial cells is increased. Two or more layers of these enlarged cells cover the wall of ducts which sometimes contain a small amount of secretion (fig. 5). Papillomatous growth into the lumen and a tumor-like infiltration is not observed. The number of mitoses does not seem to be increased.

The observations made on pair no. 62 will be described in detail. In this experiment (no. 4) the mammary glands had developed a dense system of ducts and acini when the hyp.ect. was performed. 14 days after hyp.ect. the number of ducts was reduced and acini were only occasionally to be found. 32 days after hyp.ect. the number of ducts was further reduced and the acini has disappeared, but in some places the epithelium covering the large ducts presented the changes described above. 45 days after hyp.ect. the gland was similar to those of the hyp.ect. partners of the other experiments. — In the pairs no. 100 and 134 mammary glands of the hyp.ect. partners examined 2 to 3 weeks after removing the hypertrophic ovaries revealed that the hyperplastic epithelium was persistent.

The changes observed in the mammary glands of the incompletely hyp.ect. parabionts (expts. 11—16) are remarkably different from those just described but like all those described in previous experiments of others and of my own in which growth and development is stimulated with ovarian hormones. The glands present an increased number of ducts and side buds and acini. The mammary gland of expt. 17, in which only a small number of anterior lobe cells was left after hyp.ect., shows the hyperplastic epithelium described and some normally proliferating ducts and acini. — The mammary glands of the adrenalectomized castrated parabionts remain undeveloped in all experiments.

#### *Group D: Comments.*

In a study of 5 parabiotic twins WESTMAN and JACOBSON (1944), found that the atrophy of the adrenal glands which

follows hyp.ect. is repaired when the hyp.ect. rat is united with an adrenalectomized partner. In the present investigation the hypophysis is removed from one of the twins after established parabiosis with an adrenalectomized partner. This procedure prevents the atrophy of the adrenal glands of the hyp.ect. parabiont (table 4, col. 5 and 6, and fig. 2), but it does not inhibit the hypertrophy of the ovaries (table 4, col. 4). These findings agree with those of WESTMAN and JACOBSON, and support our previous conclusion that the hypophysis of the adrenalectomized, castrated rat is secreting an excess of adrenocorticotrophic and gonadotropic hormones. The mammary glands were not examined in the 1944 investigation.

The hyperplasia of the epithelium covering the walls of large ducts (fig. 5), which regularly occurred in the mammary glands of the hyp.ect. co-twin of an adrenalectomized castrated mate must be regarded as atypical. In a large material gathered from varied experiments aimed at the study of mammary gland growth in rats I have never before observed glands built up like those of the hyp.ect. parabionts of group D. Nor was I able to find other investigators describing similar changes. However, in whole mount preparations the ducts surrounded by the hyperplastic epithelium appear very much like acini (fig. 4). Therefore, when no sections of the gland are examined microscopically, the true structure of the newly built elements may easily be overlooked.

Thus far the bearing of the mammary gland reaction just described seems to be obscure. But, as far as the problem of the present investigation is concerned the following should be kept in mind: The hypophysis of the adrenalectomized partner is secreting corticotrophin in excess, but the mammary glands of that parabiont remain undeveloped. The quantity of the corticotrophic hormones crossing into the hyp.ect. partner does not exceed those present in normal rats (the adrenal glands do not become hypertrophic). The mammary gland of the hyp.ect. parabiont, which is neither lacking corticotrophic nor adrenal cortex hormones, does not respond in the same manner as those of non hyp.ect. parabionts to the stimuli exerted by the hormones of the hypertrophic ovaries.

In incompletely hyp.ect. parabionts with hypertrophic ovaries mammary gland growth and development are promoted even when the amount of corticotrophin, secreted by the anterior lobe remnant is insufficient for preventing an atrophy of the

adrenal cortex (table 4 expt. 14). Thus neither the adrenocorticotrophic nor the adrenal cortex hormones can be supposed to play an important rôle in the normal growth of the mammary gland.

### Discussion and Conclusions.

The observations made in the present experimental enquiry into the action of the adrenotropic and the adrenal cortex hormones on the growth and development of the mammary gland can be summarized as follows (fig. 6, I—IV).

I: Group A: A hyp.ect. rat is joined in parabiosis with a partner with intact hypophysis. High doses of estradiol benzoate and a subthreshold dose of progesterone are given to the hyp.ect. parabiont. The *mammary glands* of the twins remain *undeveloped*. The *adrenal glands* of the hyp.ect. parabiont are *atrophic* (mean weight of both adrenals =  $10.2 \pm 0.05$  mg for the hyp.ect. partner, and  $58.4 \pm 2.32$  mg for the non hyp.ect. castrated co-twin).

II: Group B: A hyp.ect. rat is joined in parabiosis with a partner with intact hypophysis. High doses of estradiol benzoate are injected into the non hyp. ect. parabiont, and a subthreshold dose of progesterone is given to the hyp.ect. co-twin. The *mammary glands* of the twins become *hypertrophic*. But the *adrenals* of the hyp.ect. parabiont remain *atrophic* (mean weight of both adrenals =  $10.3 \pm 0.08$  mg for the hyp.ect. partner, and  $49.6 \pm 3.13$  mg for the non hyp.ect. co-twin).

III: Group C: An adrenalectomized rat is joined in parabiosis with a castrated mate. The ovaries and *mammary glands* of the *adrenalectomized* parabiont are *hypertrophic*. The *mammary glands* of the castrated co-twin with intact adrenals remain undeveloped (mean weight of both adrenals =  $37.1 \pm 2.41$  mg).

IV: Group D: A hyp.ect. rat is joined in parabiosis with an adrenalectomized. castrated mate. The *adrenal glands* of the hyp.ect. parabiont are *not atrophic* (mean weight of both adrenals =  $35 \pm 2.53$  mg. Sudanophobic zone absent). In spite of the presence of hypertrophic ovaries *neither duct growth nor development of acini* occur in the *mammary glands* of the hyp.ect. parabionts with non atrophied adrenals. A peculiar, *hyperplastic*, in many places *heaped epithelium* covering *large ducts* is found in all *mammary glands* of the hyp.ect. parabionts of this group. The



Fig. 1. Group B, table 2, expt. 14. Part of an atrophic adrenal gland of the hyp.-ect. parabiont. The cortex is reduced in size. Broad sudanophobic zone. Frozen section. Sudan 3 hematoxylin.  $\times 100$ .



Fig. 2. Group D, table 4, expt. 4. Part of a non atrophic adrenal gland of the hyp.ect. parabiont. Large cortex containing much sudanophilic material. Frozen section. Sudan 3 hematoxylin.  $\times 100$ .

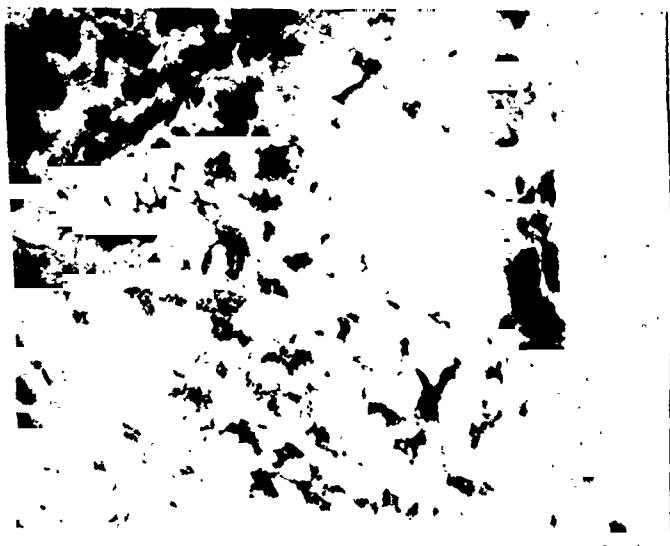


Fig. 3. Group C, table 3, expt. 15. Part of a mammary gland of the adrenalectomized parabiont. Development of ducts and acini. Whole mount. Galloeyanin chromalum.  $\times 10$ .



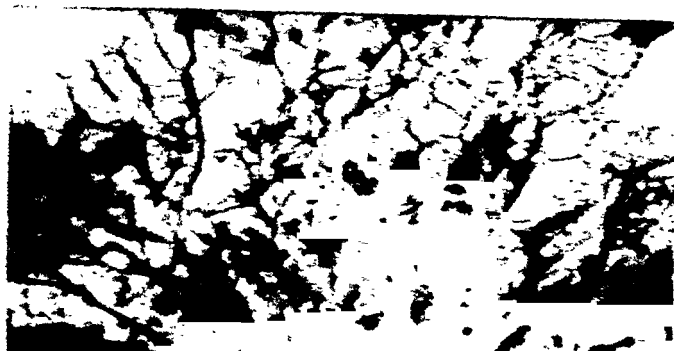


Fig. 4. Group D, table 4, expt. 9. Part of a mammary gland of the hypert. parabiont. Thickened duct walls which may give a false impression of atviti. Whole mount. Gallogranin chromalum.  $\times 10$ .



Fig. 5. Group D, table 4, expt. 10. Section of a mammary gland of the hypert. parabiont. Ducts surrounded by hyperplastic epithelial cells. Paraffin section, 5  $\mu$ . Hematoxylin eosin.  $\times 145$ .

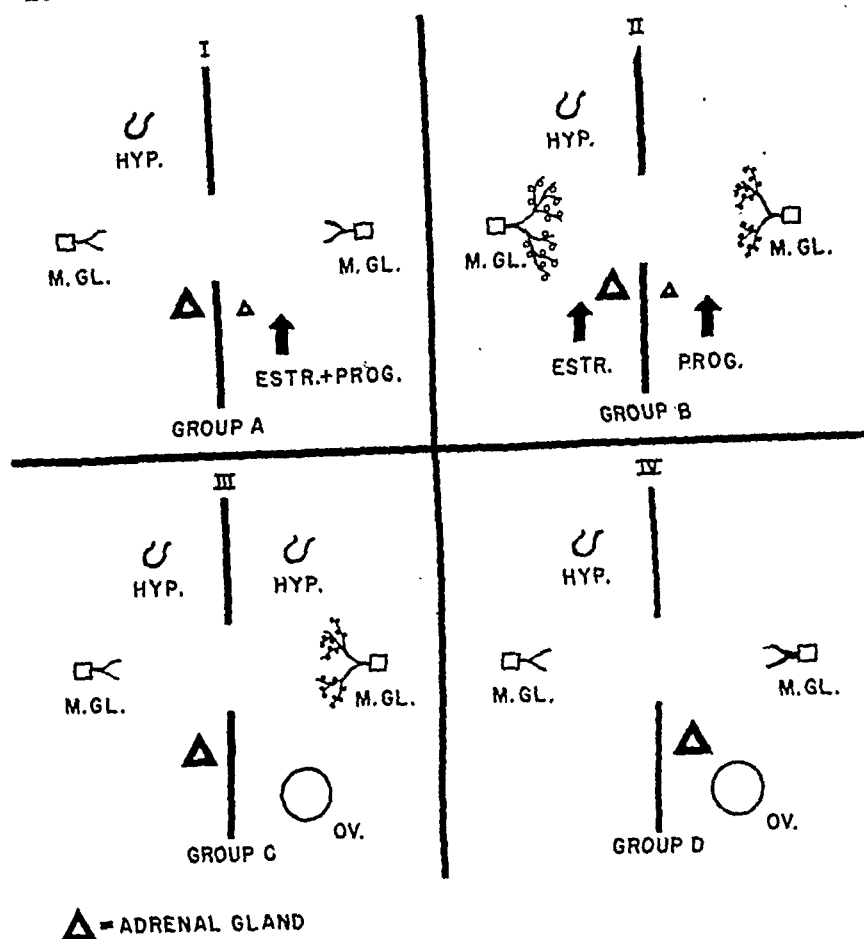


Fig. 6. Scheme illustrating the four types of experiments which elucidate that corticotrophic hormones and adrenal glands are not instrumental in normal growth and development of the mammary gland.

mammary glands of the adrenalectomized co-twin with intact hypophysis remain undeveloped.

From these results the following is concluded: *I and II (group A and B):* Mammary gland growth can be promoted in the hyp.-ect. co-twin of a partner with intact hypophysis without effecting an increased output of adrenocorticotrophic hormones sufficient for restoring the atrophy of the adrenal glands. *III (group C):* The presence of the adrenal glands is not essential for the growth and development of the mammary gland occurring in the co-twin of a castrated mate. *IV (group D):* In spite of the presence of adrenocorticotrophic hormones in quantities sufficient for maintaining the adrenal glands, a normal response of the mammary

gland to stimulation by the hormones of the hypertrophic ovaries does not occur in the hyp.ect. co-twin of a partner with intact hypophysis.

The evidence obtained from these experiments supports the view (COWIE and FOLLEY, 1947) that neither adrenocorticotrophin nor the adrenal cortex hormones play an important rôle in the process of normal mammary gland growth and development.

### Summary.

The action of adrenocorticotrophin and the adrenal cortex hormones on the mammary gland is studied on parabiotic rats.

The main findings supporting the view that neither adrenocorticotrophin nor the adrenal cortex hormones are essential for the normal growth and development of the mammary gland are:

1) In spite of a persistent atrophy of the adrenals, mammary gland growth can be promoted in the hyp.ect. co-twin of a partner with intact hypophysis injected with estrogens.

2) Ablation of the adrenal glands does not inhibit the hypertrophy of the mammary glands of the co-twin of a castrated mate.

3) When a hyp.ect. rat is joined in parabiosis with an adrenalectomized, castrated partner, the atrophy of the adrenal glands otherwise occurring after hyp.ect. is prevented. In spite of persistent hypertrophic ovaries growth and development of structures normally present in a well developed mammary gland do not occur. However, a peculiar hyperplasia of the epithelium surrounding large ducts of the mammary gland is observed. The significance of the changes induced in the epithelium is obscure.

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Table 1.

*A non hyp.ect. castrated rat is joined in parabiosis with a hyp.ecl. rat. The hyp.ecl. partner is injected with estradiol benzoate alone and later with estradiol benzoate and progesterone. Expts. on incompletely hyp.ecl. animals are included.*

Expt. "	No. of the pair	Exam. days after first inj.	Hyp.oct. partner				Castrated partner		Remnant of anterior pituit. lobe
			adrenal glands		mammary gland growth	weight mg	sudan. zone		
			weight mg	sudan. zone					
1	298 <sup>1</sup>	43	4	+	+	Ø	19	Ø	Ø
2	128	44	5	+	+	Ø	23	Ø	Ø
3	162	48	5	+	+	Ø	29	Ø	Ø
4	169	49	6	+	+	Ø	32	Ø	Ø
5	154	50	5	+	+	Ø	23	Ø	Ø
6	332 <sup>1</sup>	58	6	+	+	Ø	24	Ø	Ø
7	328	65	5	+	+	Ø	21	Ø	Ø
8	318 <sup>2</sup>	67	6	+	+	Ø	22	Ø	Ø
9	168	48	6	+	+	Ø	29	Ø	Ø
10	163	48	5	+	+	Ø	27	Ø	(+)
11	170	55	7	+	+	Ø	27	Ø	(+)
12	327 <sup>2</sup>	43	6	+	+	Ø	27	Ø	(+)
13	161	48	5	+	+	+	21	Ø	++
14	152	50	10	+	+	+	29	Ø	++
15	171	55	3	+	+	+	37	Ø	++
			3	+	+	+	22	Ø	++

(+) = small number of anterior lobe cells

++ = larger remnant of anterior lobe

+++ = large

<sup>1</sup> Treatment begun with progesterone before the hyp.ecl. partner was castrated, and finished after castration with estradiol benzoate and progesterone for a fortnight.

<sup>2</sup> The hyp.ecl. parabiont is not castrated. Ovaries hypertrophic. At different intervals injections of progesterone every other day are given for a fortnight.

Table 2.

*A non hyp.ect. castrated rat is joined in parabiosis with a hyp.ect. rat. The non hyp.ect. partner is injected with estradiol benzoate and later progesterone is given to the hyp.ect. co-twin.*

Expt.	No. of the pair	Exam. days after first inj.	Hyp.ect. partner			Castrated partner adrenal glands	
			adrenal glands		mammary gland growth	weight mg	sudan. zone.
			weight mg	sudan. zone			
1	369	34	3	++	+	15	Ø
2	364	40	6	++	+	14	Ø
3	367	40	5	++	+	25	Ø
4	116	41	9	++	+	23	Ø
5	126	46	4	++	+	21	Ø
6	127	46	4	++	+	27	Ø
7	172	55	4	++	+	29	Ø
8	92	56	6	++	+	30	Ø
9	93	56	7	++	+	28	Ø
10	111	58	5	++	+	32	Ø
11	345	62	4	++	+	29	Ø
12	103	63	6	++	+	34	Ø
13	354	69	6	++	+	23	Ø
14	362	70	4	++	+	25	Ø
			4	++	+	21	Ø
			4	++	+	18	Ø

Table 3.  
*A castrated rat is joined in parabiosis with an adrenalectomized rat.*

Expt.	No. of the pair	Exam. days after adrenalect.	Adrenalect. partner		Castrated partner	
			ovary mg	mammary gland growth	adrenals mg	mammary gland growth
1	66	14				Ø
2	68	21		+		Ø
3	67	21		+		Ø
4	63	25		+	27	Ø
5	66	29	44	+		Ø
6	68	31		+	23	Ø
7	177	31		+	14	Ø
8	178	31	158	+		Ø
9	180	31		+		Ø
10	174	33		+		Ø
11	67	39		+		Ø
12	66	44		+		Ø
13	67	52		+	17	Ø
14	177	68		+	20	Ø
15	174	70	75	+	21	Ø
16	175	70	60	+	19	Ø
17	176	71	79	+	19	Ø
18	179	72	40	+	20	Ø
19	180	72	92	+	18	Ø
			56	+	22	Ø

Table 4.

*A castrated<sup>1</sup> adrenalectomized rat is joined in parabiosis with a hyp.ect. rat. Expts. on incompletely hyp.ect. animals are included.*

Expt.	No. of the pair	Exam. days after hyp.ect.	Hyp.ect. partner				Remnant of ant. pituit. lobe
			ovary weight mg	adrenal glands		sudan. zone	
				weight mg			
1	109	25	18	14	Ø	Ø	
2	188	32	67	15	11	Ø	
3	110	42		21	14	Ø	
4	62	45	16	21	21	Ø	
5	159	59	133	18	16	Ø	
6	203	59	>100	20	18	Ø	
7	134	60	122	19	15	Ø	
8	100	61	64	26	24	Ø	
9	185	62	276	21	17	Ø	
10	187	62	59	12	11	Ø	
			75	19	18	Ø	
11	196						
12	98	34	59	13		Ø	
13	189	58	22	20	16	Ø	
14	202	58	75	18	16	Ø	
15	198	59	123	11	10	+	
16	150	59	133	17	17	(+)	
17	182	60	97	23	20	Ø	
		62	90	14	12	(+)	

<sup>1</sup> With the exception of expt. 4 in which castration was performed at parabiosis, the adrenalect. partner is castrated when the co-twin is hyp.ect.



## The Influence of Anoxia on Lactate Utilization in Man after Prolonged Muscular Work.

By

GUNNAR STRÖM.

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The blood lactate level during muscular work is chiefly dependent on the following factors:

1. Rate of formation of lactate in different tissues (mainly working muscles).
2. Rate of removal of lactate (utilization and excretion).
3. Rate of diffusion of the lactate ion. The rate of diffusion will only be of importance if it is lower than the rates of formation or removal, thus causing considerable concentration gradients between different tissues.

From previous reports (ASMUSSEN and NIELSEN 1946, LUNDIN and STRÖM 1947, ASMUSSEN, v. DÖBELN and NIELSEN 1948) it is known that in man during muscular work of both short and long duration the concentration of blood lactate has a certain inverse proportional relation to the oxygen partial pressure of the inspired air.

An insufficient oxygen supply is known to cause an increased rate of lactate formation in working muscles (cf. LUNDIN and STRÖM 1947). But anoxia might also cause changes in the other factors determining the blood lactate level, *e. g.* the rate of reconversion of lactate to glycogen in the liver. COLLEDAHL (1943) showed that during anoxia in guinea-pigs caused by severe experimental asthma bronchiale, the oxygen consumption is reduced most markedly in the liver, indicating a damaged metabolism. An impaired liver function may reduce the utilization rate of  $l(+)$ -lactate in man (SOFFER *et al.* 1937). BRINK (1936), on the other hand, found that the rate of disappearance of lactate, added to

suspensions of liver tissue from dog and rabbit, was independent of the presence of oxygen.

It is the aim of the present investigation to study the relation between the inspiratory oxygen partial pressure and the rate of lactate utilization, as calculated from the fall in blood lactate concentration during recovery from prolonged muscular exercise. The simultaneous rates of urinary lactate excretion and blood glycolysis are also determined.

### Experimental Procedure.

26 experiments were performed on two subjects, S., 70 kg body weight, and R., 90 kg, both were moderately well trained and used to work on a bicycle ergometer. The experiments were made in the morning under standardized conditions, including a light morning meal. The work was performed on a mechanical brake bicycle ergometer, with varying brake loads and a constant pedalling frequency, 60 per minute. An experiment included a work period of 20—35 min. at intensities of work of 1,260—1,440 kgm per min., followed by a recovery period of 30—60 min. The subject worked till the blood lactate level was supposed to have reached a steady state level. Immediately after the work period the subject reclined on a lounge and started breathing an oxygen-nitrogen mixture through a mouthpiece from a cylinder.

At the moment of change to low oxygen mixtures there will exist in the organism certain oxygen reserves, the most important of which is the pulmonary oxygen. These reserves can be roughly estimated to some tenth parts of a liter. The time required for equilibrium between the pulmonary air and the inspired mixture is less than 1 min., depending on the degree of hyperventilation (Boothby et al. 1948). The change to low oxygen mixtures after high intensities of work was combined with maximal hyperpnoea and great subjective discomfort.

In some experiments the subject breathed the gas mixture during the work period, with intensities of work of 720—900 kgm. per min., as well as during recovery.

Blood samples for the determination of the blood lactate level were taken from a fingertip which had been arterialised by the hand being kept in hot water (46° C). A stab was made and a few blood drops collected on a glass vessel. 0.10 ml of blood was immediately pipetted off for determination. In the first few experiments blood was drawn from an arm vein as well and subjected

to serial determinations as described below. Blood samples were usually taken after a period of rest just before the beginning of the work period, during work with 5 min. intervals and during recovery with 3—5 min. intervals. Urine was voided before and after the experiment, the volume determined and samples for lactate determination pipetted off.

### Analytical Method.

The method of lactate determination of blood and urine samples used in this investigation was as follows.

0.10 ml blood or urine is pipetted down into 1.90 ml trichloroacetic acid, containing 2 mg sodium fluoride. The precipitated protein is removed by centrifuging and 0.50 ml of the clear solution is pipetted into a stoppered test tube with 0.02 ml 4 % solution of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 3.00 ml concentrated sulfuric acid. The mixture is shaken, and the whole set of test tubes is put into a boiling water bath for 5 min., then cooled to room temperature in a cold water bath. 0.05 ml 1.5 % solution of p-hydroxidiphenyl in 2 % sodium hydroxide solution is added, the mixture shaken thoroughly and left for colour development for 30 min. at  $+20^\circ\text{C}$ . After a final heating in a boiling water bath for 90 seconds and rapid cooling to room temperature the developed colour is measured in a Coleman Junior Spectrophotometer with the spectral band at  $560\text{ m}\mu$ , using round cuvettes (Coleman 10—310). The original lactate concentration is calculated from a standard curve. In each set of determinations there are two blanks and four standard lithium lactate solutions. The error of the method including the blood sampling is less than 5 %.

The above described method of blood lactate determination is a slight modification of the colorimetric method of BARKER and SUMMERSON (1941). This method is developed from the method of MILLER and MUNTZ (1938) and is principally based on the oxidation of lactic acid to acetaldehyde by hot concentrated sulphuric acid; from acetaldehyde a violet colour is obtained by reaction with p-hydroxidiphenyl in the presence of an optimal concentration of cupric ions. BARKER and SUMMERSON recommend initial treatment of a deproteinized sample with the copper hydroxide treatment of VAN SLYKE, in order to remove glucose and certain other interfering substances that might be encountered in biological material.

EDWARDS (1938) described a simplified estimation of lactate in normal human blood, based on the iodotitrimetric method of FRIEDEMANN, COTONIO and SHAFFER (1927), but omitting the initial copper-calcium treatment recommended by these authors. The omission of the copper-calcium treatment in the method of BARKER and SUMMERSON would make it possible to make blood lactate determinations on smaller samples of blood than otherwise possible, and would also lessen the methodical variation. To investigate the necessity of the copper-calcium treatment the following experiments were made.

#### 1. Serial determinations on glycolyzed blood.

	No. of analyses	Lactic acid, mean value, mg/100 ml	Coefficient of variation, %
Without Cu-Ca treatment .....	28	129	3.4
With Cu-Ca treatment .....	28	125	3.6

#### 2. Determination of the recovery of lactic acid, added as lithium lactate to blood samples.

	No. of analyses	Lactic acid originally present, mg/100 ml	Lactic acid added, mg/100 ml	Lactic acid recovered mg/100 ml	%
Without Cu-Ca treatment .	31	9.0	25.0	24.7	99
With Cu-Ca treatment ....	6	12.9	25.0	23.2	91

#### 3. Determination of the colour intensity yielded by glucose in a solution of lithium lactate, as expressed in terms of lactic acid.

	No. of analyses	Lactic acid mg/100 ml	Glucose mg/100 ml	Colour yielded by 1 mg glucose in terms of mg lactic acid
6 .....	2.5		50	0.018
6 .....	5.0		100	0.016
6 .....	10.0		200	0.007
6 .....	20.0		400	— 0.001
6 .....	30.0		600	— 0.003

4. Serial determinations on blood samples drawn during work and urine samples were made during the experiments in this report with the original and the modified method of BARKER and SUMMERSON. No significant difference between the two methods was found.

The conclusion is drawn that under the specified conditions the initial copper-calcium treatment can be omitted.

## MEAN BLOOD LACTIC ACID

MG./100 ML.

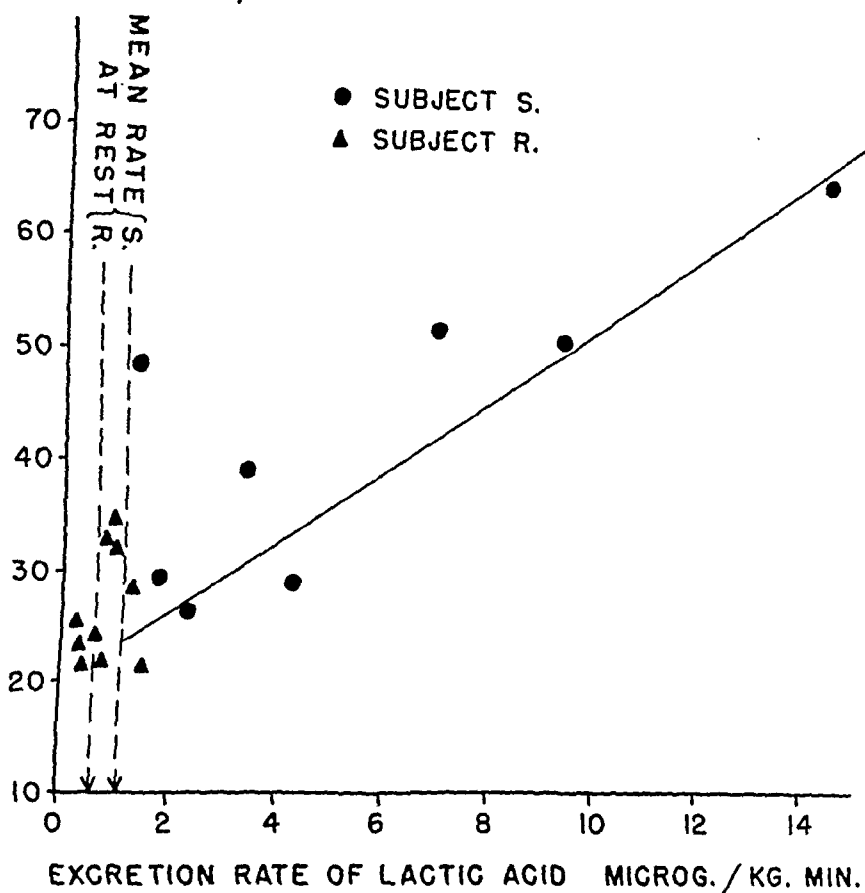


Fig. 1. Mean urinary excretion rate of lactic acid in relation to mean blood lactate concentration during work and recovery. Straight line drawn free hand.

### Result.

#### A. Urinary lactate excretion during work and recovery.

The rate of urinary lactate excretion at varying blood lactate levels at rest and during work are shown in fig. 1. Even the highest rates of lactate excretion are in both subjects remarkably low and negligible in comparison with the rates of utilization during recovery (fig. 3).

Differences in inspiratory oxygen pressure did not affect the lactate excretion rate at a given blood lactate level.

The given values during work and recovery cover periods with great changes in blood lactate concentrations. For these periods

the mean blood lactate levels were computed graphically from the blood lactate curves of the experiments. During muscular work of long duration the diuresis may decrease to 20 % of the rest value if the intensity of work is high (GRANDE and REHNBERG 1936). Subject R. usually showed a slightly diminished diuresis during the experimental period (including period of recovery), indicating a more markedly diminished diuresis during actual work. This can, however, only partly explain the fact that subj. R. often did not show any increase in urinary lactate concentration and never a marked increase, even in experiments with a blood lactate level rising up to a steady state of about 60 mg/100 ml (giving a mean blood lactate level of 30—40 mg/100 ml). In subject S. an increased urinary output of lactate was observed at high blood lactate levels, a fact that might be consistent with the opinion that in man a renal threshold exists at blood concentrations of 30—40 mg/100 ml (HEWLETT et al. 1926).

#### *B. Rate of blood glycolysis during work.*

The rate of blood glycolysis (heparin, 38° C) was determined in subject R. in 12 expts. on blood samples drawn during work, with different blood lactate concentrations. The lactate concentration in the glycolyzing sample was determined every 30 minutes for 3 hours. The rate of glycolysis was constant during this time period, and independent of the initial blood lactate level. The mean value found was 17.3 mg/100 ml per hour, meaning a lactate production of 2.9 mg per kg and min. for whole blood, or about 0.25 mg per kg and min. for the whole body. HSU (1935) found the rate of glycolysis in human blood (heparin, 37° C) to be 10—15 mg/100 ml per hour, and suggested the glycolysis rate to be similar *in vivo*. In that case blood glycolysis might markedly contribute to the blood lactate concentration at rest (cf. fig. 3). During work and recovery with high blood lactate levels this factor is quantitatively unimportant, however.

#### *C. Utilization rate of lactate during recovery.*

The fall in blood lactate concentration during recovery is shown in fig. 2. Each given value is a mean value for several experimental values, calculated in the following way. In each experiment the time is calculated that is required for the blood lactate concentration to fall between a series of arbitrary levels. The mean time values are used in constructing the mean blood concentration

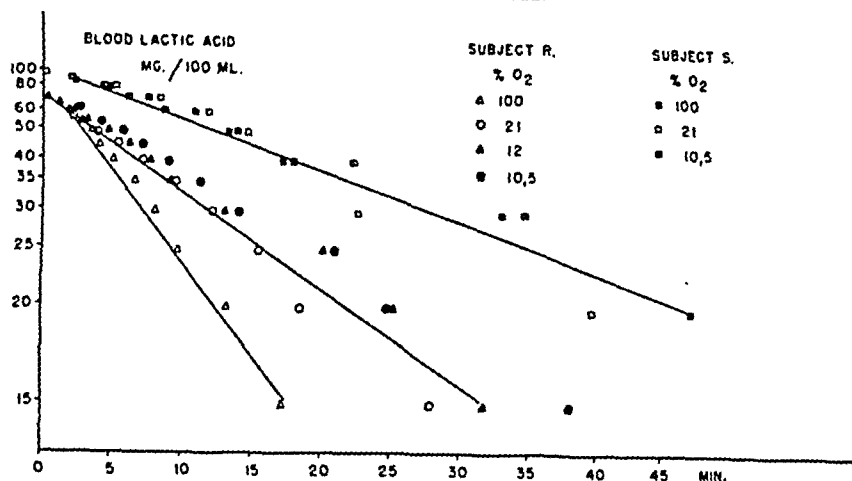


Fig. 2. Fall in blood lactate concentration after work with varying inspiratory oxygen percentages. Straight lines indicate expected relation between inverse value of lactate concentration ( $C$ ) and time ( $T$ ) if utilization processes are bimolecular.  $(C)^{-1} = \text{const.}T + \text{const.}$

curves in fig. 2, and also in calculating the mean utilization rates in fig. 3. No tendency was found that the utilization rate at a given blood lactate level was significantly influenced by the initial blood lactate level (cf. MARGARIA and EDWARDS, 1934 a). This is contrary to the conditions in galactose metabolism (DOMINGUEZ and POMERENE 1944), where after single intravenous injections of galactose the utilization rate at any given blood level diminishes corresponding to an increasing initial level.

The two subjects show different rates of lactate utilization at a given blood lactate level, subject S. showing slower rates than subject R. This can be put in relation to the fact that for a given intensity of work S. showed significantly higher blood lactate concentrations during the steady state than R. R. had a greater muscular ability and was in a slightly better state of training than S., which probably is the chief explanation to the above mentioned fact, but even working to exhaustion at high intensities of work, R. never reached the high blood lactate levels of S.

No significant decrease in the utilization rate during recovery is caused by the inhalation of 12 % or 10.5 % oxygen mixtures. This result is the same if the oxygen mixtures are administered only during recovery after high grades of work, or both during work and recovery with low grades of work.

In calculating the absolute values of lactate utilization rates, as given in fig. 3., it is assumed that at the end of work and then

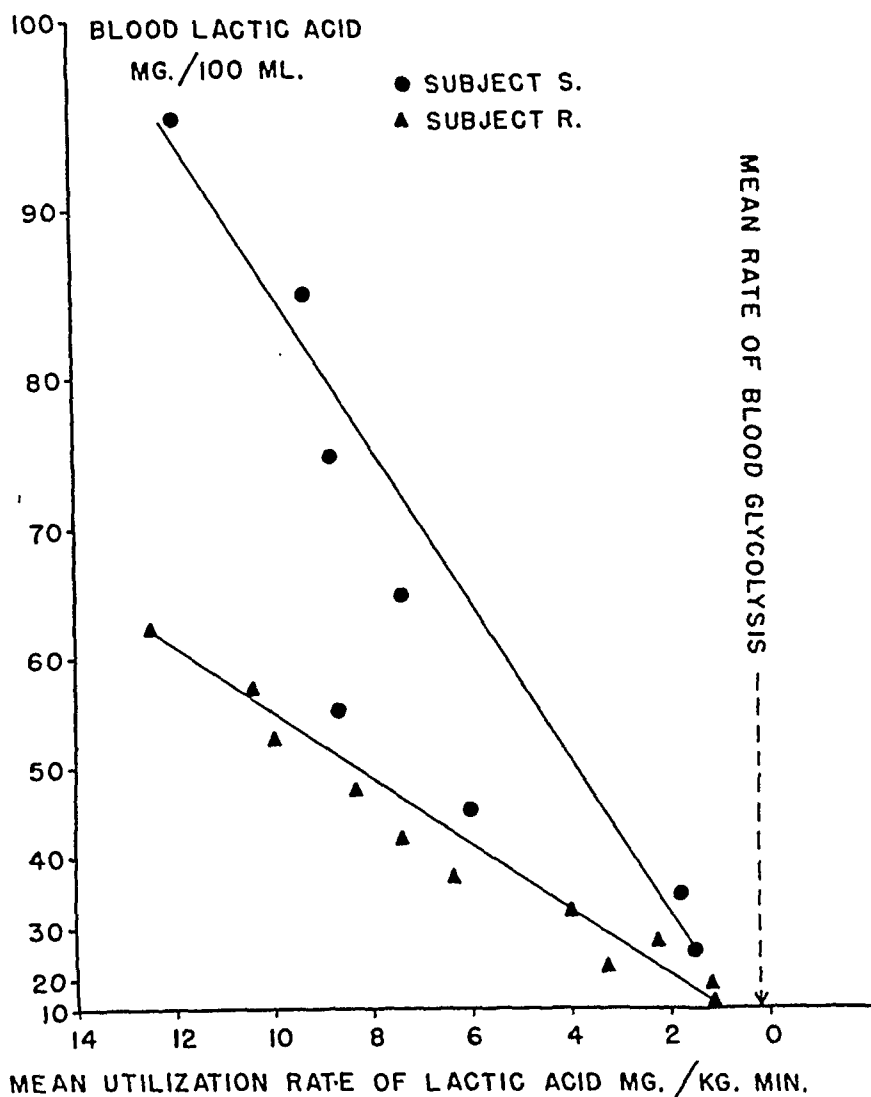


Fig. 3. Mean utilization rate of lactic acid (inspiratory  $O_2$  % = 10.5—21 %) in relation to square of blood lactate concentration. Straight lines indicate expected relation between square of lactate concentration ( $C$ ) and utilization rate ( $dC/dT$ ) if utilization processes are bimolecular. —  $(dC/dT) = \text{const. } (C)^2$

during recovery with its rather rapid alterations of lactate concentration, the blood is in equality with a distribution volume of 25 % of the body weight. This value is a minimal one, and is based on the experimental result of DOMINGUEZ and POMERENE (1944), that during rapid alterations of concentration the distribution volume for galactose is 25—30 %. This result is analogous for several other chemical compounds (ADOLPH 1943).



In subject R. the inhalation of 100 % oxygen during recovery increases the utilization rate (fig. 2), but such is not the case in subject S.

### Comment.

The result of the experiments reported in this paper indicates that the utilization of lactate is uninfluenced even by high degrees of anoxia. For a given intensity of work anoxia causes an extra rise in blood lactate level (LUNDIN and STRÖM 1946). This extra rise must therefore be due only to an increased rate of lactate formation in the working muscles. The rates of blood glycolysis and of urinary lactate excretion are shown to be unimportant in this connexion. This is further emphasized by two facts. Firstly, the absolute values of lactate utilization rates during recovery are based on the assumption that the distribution volume is 25 % of the body volume, meaning that the calculated values are minimal. Secondly, it is probable that the utilization of lactate is faster during work than during recovery. This has been shown to be the fact after short bursts of very hard work with a considerable accumulation of lactate (NEWMAN et al. 1937) and this is also the case after prolonged moderate work (RÄMMAL and STRÖM 1948).

The sweat may contain considerable amounts of lactic acid (about 80 mg %, SAIKI et al. 1931). Thus the lactate excreted in the sweat may equal or rise above that in the urine during hard work. During the experiments reported here care was taken to procure cool and dry environments, thus diminishing sweat production. Perspiration was little in subject S., moderate in subject R. It is assumed that the rate of lactate excretion in the sweat under these conditions is very small compared to the utilization rate.

HILL et al. (1924) studied the influence of variations in inspiratory oxygen percentage (13—100 %) on the removal of lactate from the body after exercise. They considered the utilization rate of lactate to be proportional to the determined extra consumption of oxygen during recovery. This extra consumption was found to be independent of the variations in inspiratory oxygen percentage. They did not determine the actual lactate concentrations, however, and later it has been shown that the assumed proportionality between lactate utilization rate and extra consumption of oxygen probably does not exist (MARGARIA et al. 1933, BARMAN et al. 1942).

In experiments with human subjects performing short work

in a decompression chamber TEPPERMAN and TEPPERMAN (1948) found that the fall in blood lactate concentration after work was the same at simulated altitudes of 1,500—4,500 m. as at sea level. The conclusions reached by HILL et al. (1924) and by TEPPERMAN and TEPPERMAN (1948) are corroborated by the result reported here.

In the graphical presentation of the experimental result in this paper (fig. 2 and fig. 3), the coordinates are chosen in such a way that if the chemical processes responsible for the removal of the accumulated lactate were bimolecular, the experimental result would give linear regression curves. The correlation to hypothetical linear regression curves is not strikingly high. The result falls into place better with the assumption that the processes are bimolecular rather than monomolecular, however. LONG (1926) concluded that in man lactate was eliminated in bimolecular processes. His experiments are open to criticism, however. LONG studied the relation between blood lactate level and extra consumption of oxygen during prolonged muscular work, assuming the extra consumption of oxygen to be proportional to utilization rate of lactate. This assumption is probably not correct (MARGARIA et al. 1933). Further, the subjects used by LONG were in different states of training, and were mainly used only in single experiments. The state of training is one of the main factors determining the blood lactate level at a given intensity of work, and also at a given oxygen consumption.

In a series of papers MARGARIA, EDWARDS and co-workers have studied the elimination of lactate after short bursts of very hard muscular work in man and the rat (MARGARIA et al. 1933, MARGARIA and EDWARDS 1934 a and b, NEWMAN et al. 1937, DILL et al. 1936). These authors find that the blood lactate concentration falls as an exponential function of time, meaning that the utilization rate of lactate is proportional to the actual lactate concentration. Such would be the case if the utilization processes were monomolecular, but also if the diffusion rate of lactate were lower than the utilization rate so that the diffusion rate were the factor to determine the rate of disappearance of lactate. The diffusion rate of lactate is considered to be very high by most authors (MARGARIA et al. 1934 a, SACKS and SACKS 1935, NEWMAN 1938), though some think that considerable concentration gradients may exist between blood and tissues (BANG 1936). Theoretically there is still left the possibility that in some tissues that are very actively utilizing lactate a considerable concentration gradient may exist. If the utilization processes were bimolecular, such concentration gradients would be likely to appear with very high lactate concentrations.

Lactate is removed in two chemically different reactions, oxidative removal and resynthesis to glycogen. It is probable that most tissues

oxidate lactate, while resynthesis takes place in the liver (MEYERHOF and LOHMANN 1926; HIMWICH et al. 1928, CORI and CORI 1929, SACKS and SACKS 1935). If the first steps in these two reactions do not follow the same chemical order, and therefore the quantitative relation between these two processes changes with changing blood lactate concentrations, the experimental result will not show a good correlation to either of the two hypothetical curves.

Finally, it can be questioned if blood lactate determinations carried out on whole blood are representative of plasma conditions. DECKER and ROSENBAUM (1942) showed that during glycolysis the plasma lactate concentration exceeds that in red blood corpuscles. JOHNSON et al. (1945) investigated the distribution of lactate in human blood. They found the plasma concentration normally higher than the corpuscle concentration, and also that the diffusion of lactate from plasma to corpuscles was a rather slow process.

These considerations lead to the conclusion, that it must be very difficult to draw definite conclusions about the chemical order of the lactate utilization processes from variations in blood lactate levels during or after muscular work. At least a very high degree of correlation between experimental result and hypothetical curve will be required.

### Summary.

A slight modification of BARKER and SUMMERSON's method of lactic acid determination is described.

The rates of lactate utilization, urinary lactate excretion and blood glycolysis during recovery from prolonged muscular exercise were studied in two subjects working on a bicycle ergometer. The results indicate that under the specified conditions

1. the rate of lactate utilization is not significantly altered by anoxia caused by inhalation of low percentage oxygen mixtures,
2. the rate of blood glycolysis is low compared with the utilization rate of lactate and is independent of the blood lactate level,

3. the rate of urinary lactate excretion is very low compared with the utilization rate of lactate, and is independent of anoxia.

The difficulties in determining the chemical order of the lactate utilization processes are discussed.

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## The Rate of Lactate Utilization in Man During Work and at Rest.

By

KALJULA RÄMMAL and GUNNAR STRÖM.

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NEWMAN et al. (1937) demonstrated that the rate of lactate removal is faster during light work than at rest. The blood lactate concentration was determined in subjects working for 1 min. at a high intensity of work followed by 45 min. of light work. The rate of lactate removal was found to increase with increasing grades of light work up to a certain limit, higher in a well-trained than in an untrained subject. This finding was corroborated by BARMAN et al. (1942), studying the lactate removal after a short period of occlusion of the blood flow to the working limbs during light work.

This investigation aims to study the influence of light muscular work on the rate of lactate utilization during a period of "recovery" after prolonged, moderately hard work, giving a steady state of blood lactate concentration.

### Method.

24 experiments were performed on two subjects, R. and S., working on a mechanical brake bicycle ergometer, with varying brake loads and a constant pedalling frequency, 60 per min. The experiments were made in the morning under standardized conditions, including a light morning meal. An experiment consisted of an initial period of rest, a work period of 25 min. at an intensity of work of 1,260 kgm. per min. (S.) and 1,440 kgm. per min. (R.)

## BLOOD LACTIC ACID

MG./100 ML

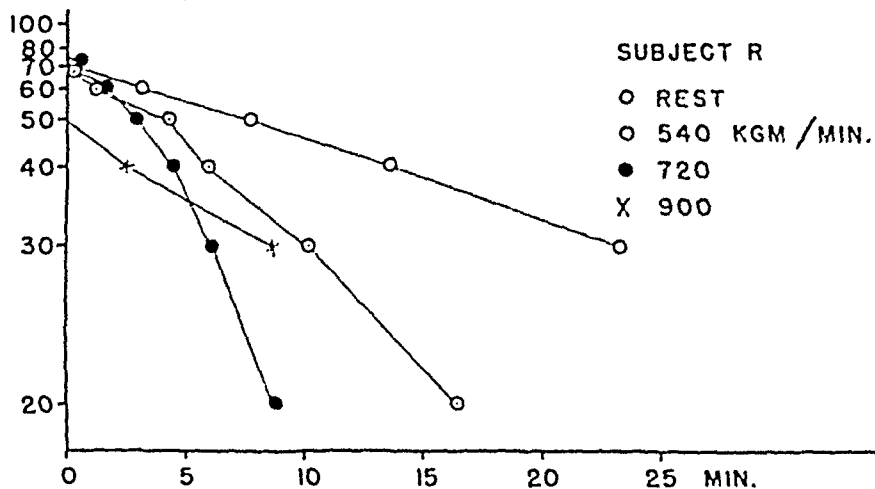
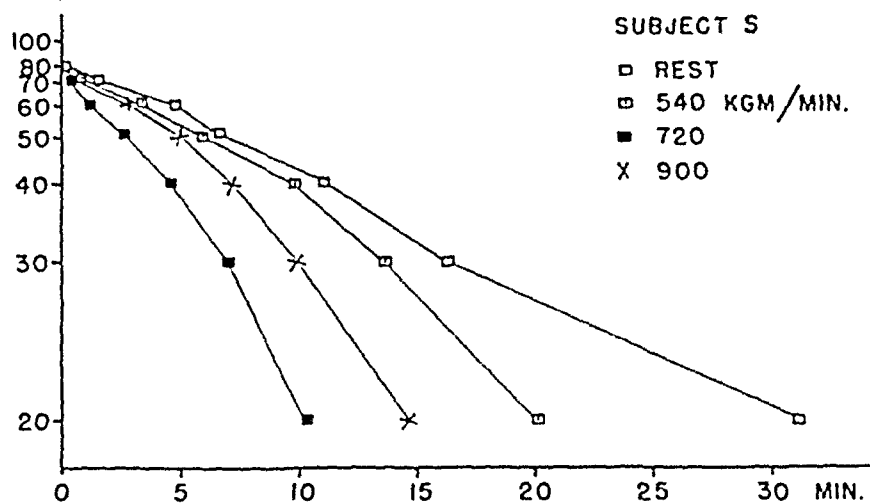


Fig. 1. Fall in blood lactate concentration after work period (25 min. work at 1,260—1,440 kgm. per min.), either at rest or during light work of varying intensity.

and finally a period of "recovery" lasting 30—60 min. with intensities of work of 0, 540, 720 or 900 kgm. per min. In a few experiments the work period lasted 2—3 min. with a work intensity of 1,980 kgm. per min., followed by a "recovery" period with 0 or 720 kgm. per min.

Blood samples for lactate determination were drawn and analyzed as described in a previous paper (STRÖM 1949).



utilization rate again decreased. Work of 900 kgm. per min. did not in itself give a raised blood lactate concentration in either of the subjects.

Confirming the result of NEWMAN et al. (1937) it was found that the lactate utilization rate after a short burst of very hard work was significantly increased with a recovery work intensity of 720 kgm. per min. as compared with a recovery rest period. A period of work at 720 kgm. per min. preceding the short burst of hard work did not influence the lactate utilization rate during recovery, however.

### Comment.

The result shows that lactate utilization rate is increased by light muscular work following a period of prolonged moderately hard work, just as after short periods of very hard work. On the other hand there seems to be a quantitative difference between the utilization processes in the two cases, as evidenced by the fact that lactate utilization is faster after prolonged work than after short work.

The effect of light muscular work on lactate utilization is further evidenced by the observation that in experiments where a subject performs light work after a period of rest, his blood lactate level during work may significantly sink below his rest value (ASMUSSEN 1946).

About the causes of the effect of light muscular work on lactate utilization, NEWMAN et al. (1937) propose two possible factors:

1. An increased cardiac output and muscular blood flow, giving an increased rate of transportation of lactate from inactive to actively utilizing tissues.

2. An increased utilization (oxidation, cf. SACKS and SACKS 1935) of lactate in the working muscles.

That the last-mentioned factor really plays a rôle seems to be indicated by the fact that the hastening effect of light work on lactate utilization has passed its maximum already at intensities of work that do not in themselves give a raised blood lactate concentration.

### Summary.

The fall in blood lactate concentration during "recovery" from prolonged, moderately hard muscular work was studied in two



subjects, working on a bicycle ergometer. The rate of lactate utilization during "recovery" was found to be greater during light muscular work than at rest. Lactate utilization at rest was found to be faster after prolonged than after short muscular work.

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ON THE MODE OF ACTION  
OF OVARIAN HORMONES ON  
GROWTH AND DEVELOPMENT  
OF THE MAMMARY GLAND

BY

DORA JACOBSON

LUND 1948



The experiments of LANE-CLAYTON & STARLING in 1906 drew attention to humoral factors as possible stimulators of the development and function of the mammary gland. Later, concomitantly with the gradual evolution of the hormone theory, intensive studies were made on the endocrine control of the mammary gland.

Many investigators have been concerned with the action of the ovarian hormones on the mammary gland. The stimulating effect of estrogens on the growth of ducts, and in some species on the development and secretory activity of ducts and acini has been demonstrated (for lit. see TURNER, 1934, 1939, NELSON, 1936, FOLLEY, 1940, PARKES, 1944, DYRENDahl, 1946).

However, in spite of a large number of investigations the mode of action of the estrogens on the mammary gland is not clearly understood.

Authors who have succeeded in producing mammary growth in hypophysectomized (hyp. ect.) animals treated with estrogens postulate a direct stimulating action, similar to that on the vagina and uterus (for. lit. see FREDRIKSON, 1939, RIDDLE, 1940, FOLLEY, 1947). The situation as to a direct effect of estrogens, remains obscure since a large number of investigators found that estrogen administration did not evoke mammary development in the hyp.ect. animal.

ASTWOOD, GESCHICKTER & RAUSCH, 1937, suggest that the failure of the mammary gland of the hyp.ect. animal to respond to administration of estrogens is due to the poor general condition prevailing after hypophysectomy. On the other hand work on hyp.ect. animals treated with pituitary hormones either alone or in combination with steroid hormones, suggests the involvement of an anterior pituitary factor (or factors) in the process of mammary development.

On the basis of a considerable number of investigations, summarized by LEWIS & TURNER, 1939, and by MIXNER & TURNER 1943, the hypothesis is advanced that the ovarian hormones act upon

the hypophysis, releasing specific mammogenic factors, Mammogen I and II, which in turn stimulate the growth of ducts and alveoli.

But in view of the observation that the effect of percutaneous estrogens on the mammary gland is confined to the region of application, other workers, unable to reproduce results supporting the hypothesis of TURNER et al. conclude that the ovarian hormones act directly on the mammary gland. This action is assumed to be enhanced synergistically by one or more of the known pituitary hormones (HISAW & ASTWOOD, 1942).

A detailed review of the conflicting experimental work and of the controversial views is given by FOLLEY, 1940, 1947, RIDDLE, 1941, PETERSEN, 1942, 1944, MEYER, 1945, HOOKER 1946 and SPEERT 1948. Some of the difficulties encountered in investigations on this subject will be mentioned.

As COWIE & FOLLEY, 1944, 1947, and RICHARDSON, 1947, pointed out it is not possible to measure the mammary growth objectively. The size of the different glands varies and the microscopic picture is not quite uniform in one and the same animal. If the changes observed are confined to a slight increase in the structures present before treatment, the study of one gland from each animal may give rise to misleading conclusions.

The reaction of the mammary gland is influenced by the general condition of the animal. It is widely agreed therefore that the hyp. ect. animal is no ideal test object. Nevertheless — but in view of the problem, naturally — evidence chiefly from studies on hyp. ect. animals treated with hormones constitutes the basis of our knowledge of the hormonal control of mammary gland growth.

In the present investigation observations were made on parabiotic rats. This technique has the advantage that the hyp. ect. co twin of a partner with intact hypophysis is in a better condition than the single rat with its pituitary body removed, and that the exchange of hormones between the two partners is such that the experimental conditions can be suitably varied.

An extensive discussion of the exchange of metabolites between two partners of parabiotic twins is outside the scope of the present investigation. The reader is therefore referred to the papers of RANZI & EHRLICH, 1909, CHRISTEA & DENK, 1910, SCHMIDT, 1922,

PFEIFFER, 1929, LOWER & HICKEN, 1932, HILL, 1932, MØLLER-CHRISTENSEN, 1932, 1935, DU SHANE et al, 1935, GROLLMAN & RULE, 1943, SIBLEY & HUGGINS, 1946. The basic concepts relevant for the interpretation of the results presented in this paper are as follows: if pairs of rats are joined in parabiosis with coelio-anastomosis, the procedure used in the present investigation, the chief communication is the lymphatic system, the common peritoneal cavity and the tissue spaces at the anastomosis. Metabolites readily diffusible into the lymphatic system as for example carbohydrates (LOWER & HICKEN, 1932, SHIPLEY & MEYER, 1947) are quickly transferred from one parabiont to the other. Vascular connections are poorly developed. Humoral agents transported by the blood stream exert their action in the recipient only if they are constantly present in the circulating blood of the donor rat in excessive amounts, thus allowing the passage of quantities necessary for inducing specific effects in the recipient (e. g. SCHMIDT, 1922, HILL 1932, DU SHANE et al, 1935). Nervous connections are apparently lacking (RANZI & EHRLICH, 1909).

In the present investigation the reaction of the mammary gland of the hyp. ect. rat united parabiotically with a non hyp. ect. partner is studied.

## Experimental.

Virgin female albino rats with a few exceptions from an inbred strain kept at our department, and fed on table scraps, milk and grain ad libitum, are used. Apart from controls on single animals all the experiments are performed on parabiotic rats.

The parabiosis is made on two littermates, usually when 21 days old; details concerning the age see below. The technical procedure is a slight modification of that used by other workers (e. g. HILL, 1932). Under ether narcosis, after removing a piece of the skin along one side of the body, and after opening the abdominal cavity of each animal the abdominal walls and skin are united by suturing them together. The twins finally have a common abdominal cavity. The skin suture extends from the hindleg to the ear in such a way that the animals are joined closely together. By this means the gradual development of a stalk at the junction of the two animals is avoided. Neither torsion (BUNSTER & MEYER, 1933, MØLLER-CHRISTENSEN, 1935) nor

spontaneous separation occurs. It is not necessary to operate under sterile conditions, but the wound edges have to be carefully adapted, and any kind of damage to the tissues or bleeding has to be avoided. Displacement and adhesions of the intestines occurred very rarely.

The mortality, due to the well known (e. g. FELS, 1929) parabiosis intoxication is very high and unpredictable. Since in my experience intoxication occurs usually about a fortnight after the animals have been united, only such twins are included in the material, which have been in an excellent condition until the termination of the experiments. The duration of most of the experiments is 2 to 4 months. When possible several experiments are made on one and the same pair. The total number of parabiotic pairs used in this study amounts to 81, the maximum lifetime being 8 months.

In most of the experiments one or both partners are castrated, and one partner is hyp ect.; details are given when the different types of experiments are described.

Hypophysectomy is performed in some cases before, and in all others after parabiosis has been established. A modification of the method described by COLLIP, SELYE and THOMSON, 1933, is applied. The completeness of the hypophysectomy is controlled by microscopic examination of serial sections throughout the pituitary capsule and adjacent tissues.

Animals treated receive 10 or 100  $\mu$ g Di Menformon\*) (= estradiol benzoate in olive oil) intramuscularly, diluted so that with a few exceptions not more than 0,02 ml have to be injected. In some groups 5 mg crystalline progesterone\*) dispersed in about 2 ml 0,9 per cent saline solution is administered subcutaneously.

The mammary glands are either extirpated during the course of the experiment or taken at autopsy. Care has been taken to examine corresponding glands of the two animals, and to avoid those glands situated near the junctional area. Each mammary gland is stretched on tracing paper, fixed in Carnoy's solution, stained with gallocyanin chromalum (introduced by EINARSON, 1932, and used for various tissues by LAGERSTEDT, 1947, 1948), cleared in benzene and mounted

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\*) Di Menformon was kindly supplied by the Pharmacia Ltd., Stockholm, Progesterone by the Nordiska Organon Ltd., Stockholm.

in toto in Canada balsam. At a pH of about 2.0 the parenchyma stains dark blue, whereas the surrounding connective and muscular tissue remains faintly coloured. The selective and progressive gallo-cyanin staining allows a direct examination of the mammary gland without interfering with the parenchyma. The usually applied laborious technique of freeing the gland from the surrounding tissues is not employed. Histological details are studied on hematoxylin-eosin stained paraffin sections 5—10  $\mu$  thick. Generally the whole mount preparation shows the area taken for the histological examination.

In some experiments at the beginning of this investigation either the gland was embedded in paraffin and cut into serial sections, or whole mounts, or 300  $\mu$  thick frozen sections were prepared, the latter stained with Delafield's hematoxylin. Fixing of these glands was done with 10 % formalin.

A total of 350 glands of parabiotic rats have been examined.

The type and the degree of the glandular development is given in an abbreviated manner by using a scheme devised by COWIE and FOLLEY, 1947:

*"Arborescence of duct system.*

- D — Restricted duct system showing little branching; a few tertiary ducts present.
- 2D — Duct system of moderate extent showing intermediate degree of arborescence; many tertiary branches and also some of higher order.
- 3D — Widespread duct system, showing high degree of arborescence.

*Club-shaped end buds, usually deeply staining.*

- E — A few present.
- 2E — Club-shaped end buds regularly disposed round the periphery.

*Side buds.*

- B — Very few present.
- 2B — Numerous side buds fairly evenly distributed.
- 3B — Ducts covered with side buds.



*Alveolar development .*

- A — Occasional alveoli present.
- 2A — Moderate alveolar development, evenly distributed.
- 3A — Ducts covered with alveoli.”
- In addition side buds present only in the central part of the gland are marked by "c", and the amount of secretion is characterized as follows:
- S — Little secretion in some of the ducts or alveoli.
- 2S — Ducts and alveoli distended with secretion.
- 3S — Ducts and alveoli widely distended with secretion.

In most experiments the circumference of the area covered by the mammary gland (*area*) is measured by means of a planimeter (1 : 100 000) after the whole mount has been prepared. As the area of glands from different regions differs, and as the material consists of single glands, only corresponding glands can be matched. The comparison of the areas of two corresponding glands from the parabiologic twins illustrates whether the gland from one partner is more developed than that of the co twin. The error of these measurements is quite large as it depends on the degree of stretching of the gland while making the whole mount preparation.\*)

The scheme employed in tabulating my observations serves the purpose of presenting the whole material. The bearing of the figures given in the tables to qualify the growth and development of the glands will be clear from the illustrations and is commented on when describing the experiments. Conclusions are drawn only from observations of changes which are obvious and evidently proving a specific effect. A statistical evaluation was therefore considered unnecessary.

The experiments of the present investigation are divided into four groups. These will be reported and discussed separately before comparing them with each other.

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\*) The author is aware that the method employed in scoring the degree of mammary gland development is rather rough; the accuracy, however, is sufficient for the purpose of this investigation.

## Results.

### Group A.

*A castrated female rat is joined in parabiosis to a partner with ovaries and pituitary body left intact* (fig. 14, 1). The results of the experiments belonging to group A are summarized in the tables 1, 2 and 3.

Table 1 includes 14 mammary glands from the non castrated partner and 13 glands from the castrated partner of 10 parabiotic twins.

Castration has been performed 0 to 18 days before parabiosis. The two animals have been united together at about 60 days of age. —After 11 to 87 days the ovaries and the mammary glands of the non castrated animals present the well known hypertrophy. The mammary glands show ducts covered with acini containing more or less secretion (fig. 4). With the exception of pair no. 50 in which occasional acini were observed, the mammary glands of the castrated parabionts are as undeveloped as those of untreated single castrated female rats, but the number of side buds seems to be slightly increased in some cases.

Table 2 summarizes 10 observations made on the mammary glands of 8 parabiotic rats, united when 20 to 28 days old. In expts. 1 and 6 one animal has been castrated a fortnight after the parabiosis operation, in the other experiments after 4 to 5 months. The mammary glands examined 21 to 53 days after castration (expts. 3 to 10) show hypertrophic changes in the non castrated partner. Here the proliferation of secreting ducts is more prominent than the development of acini. The weight of the ovaries is not very much increased, and with exception of expts. 2 and 7 large follicles but no corpora lutea are present. The mammary glands of the castrated partners seem to be slightly more developed than those of single castrated rats.

Table 3 shows the reaction of 23 pairs of mammary glands from 14 parabiotic twins. The parabiosis is performed at the age of 21 days. In these cases a fortnight after the animals have been united one partner is castrated. At the same time a sham hyp.ect., leaving a remnant of anterior pituitary intact, is done on the other partner.

10 to 22 days after castration the examination of 8 mammary glands of sham operated partners reveals a not very extensive, but distinct

proliferation. 26 to 61 days after castration hypertrophic ovaries with large follicles and corpora lutea, and hypertrophic mammary glands with ducts covered with side buds are present (fig. 1). Alveoli occur in 11 of the mammary glands examined. Only in 5 experiments secretion is observed. -- The mammary glands of the castrated co twins remain undeveloped.

### Group A: Comments.

The observation of the development of hypertrophic ovaries and mammary glands in the non castrated co twin of a castrated mate, as well as the non-reaction of the mammary gland of the castrated parabiont is in accord with earlier investigations (e. g. ZECKWER, 1944). It supports the current view that the increased amount of gonadotrophic hormones released from the anterior pituitary body of the castrate (e. g. EMERY, 1932) produces the ovarian hypertrophy which in turn causes the hypertrophic changes in the mammary gland. There is an excessive release of hormones from the hypertrophic ovaries. But the amount of ovarian hormones passing into the castrated partner is below the threshold needed to regulate the secretion of gonadotrophic hormones from the anterior pituitary body, and also too small to stimulate the mammary gland growth.

Compared with the mammary glands from the experiments in which castration is performed before uniting the animals (table 1) the gland of a non castrated parabiont united with a partner castrated after established parabiosis (table 2) presents another type of hypertrophy. In the experiments summarized in table 2 the animals were considerably older when castrated (about 5 months) than those of table 1 (about 40 days). It is felt that the difference in the induced structural changes may be referred to the age at castration. This assumption is supported by the result of the experiments of table 3. Here too, castration is performed after the animals have been united, the age at castration being about the same as in the experiments of table 1. The type of hypertrophy found in the ovaries as well as in the mammary glands is similar to that reported for the experiments of table 1. The experiments included in table 3 show further, that the mammary gland hypertrophy occurring after an incomplete hypophysectomy is the same as that developing in the non hyp. ect. rat twinned with a castrated female.

### Group B.

In the following group B the effect of a complete hypophysectomy on mammary gland growth is studied in parabiotic rats under the same conditions as in group A. — *A castrated female rat is joined in parabiosis to a partner with ovaries left intact, but with the hypophysis removed* (fig. 14, II).

The results of the experiments of group B are summarized in table 4 and 5.

Table 4 shows the reaction of 27 mammary glands of 17 hyp.ect. rats and of 22 mammary glands of the castrated co twins. The parabiosis is performed when the animals are immature. About a fortnight after parabiosis one partner is castrated and the other one hyp.ect.

The mammary glands studied at different intervals from the 5th to the 62nd day after hypophysectomy and castration present a roughly uniform picture (fig. 2 and 3) both in the hyp.ect. and in the castrated parabiont. In spite of the presence of ovaries with the well known hypertrophy of the follicular system and with corpora lutea in 8 experiments, the mammary glands of the hyp.ect. parabionts remain undeveloped. No proliferation of ducts and acini occurs. However, end buds and occasional side buds are present in the mammary glands both of the hyp.ect. and of the castrated parabionts. The duct system is somewhat more widespread in the glands from the non hyp.ect. castrated mates.

Table 5 includes 13 experiments performed on 10 pairs of rats united when 40 to 50 days old. One of the two partners was castrated 0 to 17 days previous to the parabiosis operation. 13 to 20 days after the parabiosis operation the other partner is hyp.ect. The mammary glands are examined at different intervals, from 2 to 73 days, after hypophysectomy.

At the beginning of the experiment, that is at hypophysectomy of the non castrated partner, the ovaries and mammary glands present the hypertrophic changes reported in group A (table 1). 6 days after the hypophysectomy a regression of the alveoli is observed, and from the 14th day after hypophysectomy onwards, the alveoli and most of the side buds disappear (fig. 4 and 5). Against the gradual atrophy of the mammary glands the established hypertrophy of the ovaries in-

creases further. The ovaries studied contain a great number of large follicles and apparently well developed corpora lutea (fig. 6). The mammary glands of the castrated co twins remain unchanged with a more or less bare system of ducts.

### Group B: Comments.

The experiments of group B confirm previous observations of hypertrophic changes demonstrated in the ovaries, uterus and vagina of a hyp.ect. rat united parabiotically to a castrated, non hyp.ect. partner (e. g. DU SHANE, LEVINE; PFEIFFER & WITSCHI, 1935, MØLLER-CHRISTENSEN, 1935). However, in the hyp. ect. parabiont the excessive release of hormones by the ovaries neither stimulates the growth of the undeveloped mammary gland (table 4), nor prevents the involution of the mammary gland with a well developed system of ducts and acini (table 5). The mammary gland of the hyp.ect. parabiont shows a close resemblance to that of the castrated co twin (fig. 2 and 3). It does not present the excessive atrophy (fig. 7) known to occur in single hyp.ect. rats (cf fig. 2 and 7). Therefore, and with regard to the conditions regulating the exchange of metabolites in parabiotic twins (see page 5) the unresponsiveness of the mammary gland of the non castrated, hyp.ect. parabiont in group B can hardly be explained by a general adverse effect of the hypophysectomy, only.

In the experiments summarized in table 4, where the ovaries were immature at hypophysectomy, the occurrence of corpora lutea was only rare, which agrees with earlier investigations (e. g. WITSCHI and LEVINE, 1934). The ovaries referred to in table 5 present a large number of microscopically intact corpora lutea, most of them apparently preserved by the action of the overstimulated follicles (WESTMAN, 1934, ROBSON, 1937, WESTMAN & JACOBSSÖHN, 1937).

According to DU SHANE et al. the hypertrophic ovaries of a hyp. ect. co twin of a castrate are secreting far less than 150 rat units of estrogen daily "one may venture an estimate of 5 to 20 daily units".

### Group C.

In this group I investigated, whether a response of the mammary gland can be obtained by injections of estrogen and progesterone into the hyp. ect. partner using doses considerably exceeding those just

mentioned. — *A castrated female is joined in parabiosis to a hyp.ect. rat. The hyp.ect. parabiont is treated 1) with estradiol benzoate alone, 2) with estradiol benzoate and progesterone.* (Fig. 14, III)

The experiments included in group C<sub>1</sub> are summarized in tables 6 and 7.

Table 6 shows 10 experiments performed on 7 parabiotic rats. The hyp. ect. partner is injected with 10 µg estradiol benzoate every other day. The mammary glands are examined at different intervals from 14 to 32 days after the first injection.

At hypophysectomy the mammary glands of nos. 53 and 58 are fully developed, as the co twin had been castrated 21 days previously. Involution occurred following hypophysectomy (see table 5, expts. 6 and 7). At the beginning of the treatment only remnants of ducts and acini are left. The mammary glands of all the other animals had not been stimulated to additional growth before the experiments were begun.

After treatment the mammary glands of the hyp.ect. partners are similar to those of table 4 and 5. With the exception of no. 4 the weight of the ovary exceeds that of a single hyp.ect. rat by about 8 up to more than 30 times. The mammary glands of the castrated partners are in most cases slightly more developed than those of their hyp. ect. co twin.

Table 7 shows 6 experiments done on 6 pairs used for the foregoing table. The dose of estradiol benzoate is increased tenfold. Otherwise the conditions are the same as those of the experiments of table 6.

The degree of mammary gland development in the hyp.ect. partner is roughly the same as that observed in the experiments of table 4, 5 and 6 (fig. 8). Only in no. 58, expt. 1, in which the mammary gland had been developed previously, the number of side buds is increased. The weight of the ovaries exceeds those of single hyp.ect. rats by about 2 up to 25 times. The mammary glands of the castrated partners are in 3 cases slightly more developed than those of table 6.

The results of experiments included in group C<sub>2</sub> are summarized in tables 8 and 9.

Table 8 shows 7 experiments. 10 µg estradiol benzoate and 5 mg cryst. progesterone are given to the hyp.ect. parabiont during a period

of two to three weeks. The mammary glands of the hyp.ect. animals present a roughly uniform picture. In the central part a thickening of ducts and a slightly increased number of side buds is observed. The peripheral ducts are thin with occasional side buds only. Acini are absent in all glands examined (fig. 9). — The mammary glands of the non hyp. ect. partners show a slight growth of the duct system and in some cases a slightly increased number of side buds. Secretory processes are not observed.

Table 9 includes 14 experiments performed in the same way as those of table 8 except that the dose of estradiol benzoate is increased tenfold. In expts. 6, 7 and 8 the microscopic examination of the contents of the pituitary capsule reveals a small number of anterior lobe cells. A larger amount of anterior lobe tissue is left in expts. 9—14.

The mammary glands removed from the hyp.ect. partners of expts. 1, 2, 4, 5, 7, 8 are slightly more developed than those of the hyp.ect. partners of table 7 and 8. They present a number of evenly distributed side buds but no acini (fig. 10). The mammary glands of the incompletely hyp. ect. animals of expts. no. 9 to 14, however, are more or less fully developed with ducts covered by a large number of evenly distributed side buds and acini, distended with secretion in expts. no. 12 to 14.

A proliferation of ducts, mostly with secreting cells, is found in the mammary glands taken from the non hyp. ect. partners of the parabioc pairs.

### Group C: Comments.

The result of the experiments included in tables 6 and 7 shows that the mammary gland of a hyp.ect. co twin of a partner with intact hypophysis does not respond adequately to estrogen given in doses more than 10 times in excess of those released from hypertrophic ovaries. Controls on single castrated rats with intact hypophysis showed the type and degree of proliferation demonstrated by others to occur after treatment with increasing doses of estrogens. Therefore, and with regard to the observations made in group A, it seems reasonable to conclude that the non responsiveness of the mammary gland of the hyp.ect. co twin of a partner with intact hypophysis can neither be explained by an insufficient administration of estrogen nor by an insufficient release of hormones from the hypertrophic ovaries.

The ovaries of the hyp. ect. partner injected with estrogen (group C<sub>1</sub>) remain hypertrophic. Only a slight proliferation of ducts is found in the mammary gland of the non hyp.ect. partner. Thus, the amount of estrogen passing into the non hyp. ect. parabiont is insufficient 1) to inhibit the output of gonadotrophic hormones from the anterior pituitary, and 2) to stimulate mammary gland growth significantly.

Administration of progesterone in addition to the injections of estradiol benzoate (group C<sub>2</sub>) results in a slight modification of the mammary gland reaction just described. This modification of the response is greater with 100 µg estradiol benzoate than with 10 µg. In the experiments of table 9 the mammary glands of the hyp.ect. co twins of non hyp.ect. mates present proliferative changes in 6 of 8 cases (fig. 10). A proliferation of ducts, in 5 cases distended with secretion, is found in the mammary glands of the non hyp.ect. parabionts.

Here the amount of estrogens passing into the non injected partner is apparently larger than in the experiments performed without progesterone (table 7). It is felt that this observation supports ZECKWER. 1946, who states that more estrogens are passing into the castrated co twin of a female rat during the stage where the hypertrophic ovaries contain many large corpora lutea.

This assumption is supported by the experiments of table 9a showing an analogous result as those of table 9. In the experiments of table 9a progesterone is given to the hyp. ect. co twin of a non hyp.ect. castrate. As in the experiments of group B table 4 the ovaries of the hyp. ect. parabiont are hypertrophic. But after the administration of progesterone to the hyp. ect. parabionts of table 9a the mammary glands of both partners are more proliferated than those of table 4.

It should be kept in mind that a proliferation of ducts of the mammary gland of the hyp.ect. partner is only observed in twins where growth processes are found in the mammary gland of the castrated partner with intact hypophysis as well. This point will be considered later (page 24).

Of interest is the complete absence of acini and, with regard to the doses employed, the extremely slight degree of duct growth observed in the hyp. ect. parabiont. The refractoriness of the mammary gland



of the hyp. ect. parabiont is obvious especially when contrasted with the fully developed glands of controls with a remnant of secreting anterior lobe tissue left after hypophysectomy (table 9, expts. 9—14, table 9a, expts. 9—13).

The striking difference between group A (fig. 4) on the one hand, and B (fig. 2) and C (fig. 8) on the other, with respect to the reaction of the mammary gland supports the view that in addition to the ovarian hormones, complementary stimuli originating in the anterior pituitary are necessary for the growth and development of the mammary gland. Further, the "sensitizing" pituitary factor (GARDNER & WHITE, 1941, REECE & LEONARD, 1941), if present in the non hyp. ect. partner, is not released in amounts sufficient to exert a definite action on the mammary gland of the hyp. ect. co twin.

In a preliminary report of observations from experiments on parabiotic rats treated with 10  $\mu$ g estradiol dipropionate GRANGER, 1945, postulates the presence of a specific mammogenic factor crossing from the partner with intact hypophysis to the hyp.ect. co twin. However, no detailed account concerning the age (23 to 46 days) of the animals, the beginning of the injections after hyp.ect., and the site of the injections ("usually" the hyp.ect. partner) is given. According to LEONARD, 1943, estradiol dipropionate causes growth of the end buds in hyp. ect. single rats if their weight is less than 70 gms. and the treatment begun immediately after hyp. ect. LEONARD discusses his observation "The influence of age and body size on the growth response of the mammary glands of the hyp.ect. rats suggests that the component tissues possess certain potentialities for hyperplasia which can be activated by estrogens during certain periods of immaturity. That this ability to respond to estrogens is subsequently lost was indicated by the absence of mammary growth in the larger and older animals." A further analysis of GRANGER'S observations might reveal an agreement between her results and those of the present investigation.

### Group D.

According to one of the theories mentioned on page 3 the ovarian hormones exert their action on the mammary gland via the hypophysis. In the experiments reported below I have therefore investigated the reaction of the mammary gland of the hyp. ect. rat joined

parabiotically to a non hyp. ect. partner which is treated with estrogen. — *A castrated female rat is joined in parabiosis to a hyp. ect. partner. The castrated non hyp. ect. partner is treated with estradiol benzoate, the hyp. ect. co twin is 1) either left untreated or 2) injected with progesterone.* (Fig. 14, IV)

The experiments included in group D<sub>1</sub> are summarized in the following tables 10 and 11.

Table 10 shows 9 experiments. For about a fortnight the non hyp. ect. parabiont is injected with 10 µg estradiol benzoate every other day, the hyp. ect. partner is left untreated. The non hyp. ect. parabiont of expts. 1—5 is castrated when the injections are begun. In expts. 6—9 the castration of the non hyp. ect. partner is performed simultaneously with the hypophysectomy of the co twin. Thus at the beginning of the experiments the mammary glands of the hyp. ect. partners are in all cases undeveloped.

At the end of the experiment the mammary gland of the hyp. ect. partner is undeveloped. It shows a slight proliferation of side buds and a thickening of ducts only in expts. 4, 6 and 8. In all but 2 cases (expts. 6 and 9) the ovarian hypertrophy described above (group A and B) is greatly reduced. The mammary glands of the non hyp. ect. partners present the proliferation of secreting ducts demonstrated by others in single non hyp. ect. rats after treatment with high doses of estrogens.

Table 11 summarizes the results obtained under the same conditions as before (table 10) except that the non hyp. ect. partner is injected with 100 µg estradiol benzoate. The experiments of table 10 are continued for about a fortnight with the 100 µg dose of estradiol benzoate.

The changes observed are in 6 of the 8 cases similar to but more pronounced than those described for expts. 4, 6 and 8 of table 10. The number of evenly distributed side buds in the mammary gland of the hyp. ect. animal is more increased (fig. 11). In expt. 3 numerous side buds consisting of ducts containing secretion are present. It is difficult to distinguish these ducts from true acini. — The ovaries present are atrophic. — The mammary glands of the castrated partners show widely distended ducts containing much secretion.

The results of the experiments included in group D<sub>2</sub> are summarized in the following tables 12 and 13.

Table 12 includes 10 experiments on 8 parabiotic rats. One partner is castrated, the other is hyp. ect. and castrated. The non hyp. ect. castrate is treated with 10  $\mu$ g estradiol benzoate every other day (as in the expts. of table 10), the hyp. ect. co twin is injected with 5 mg cryst. progesterone every other day. At different intervals from 14 to 33 days after the beginning of the treatment a mammary gland of each parabiont is examined.

The mammary glands of the hyp. ect. partners remain undeveloped. In 5 cases the number of side buds is increased, but only in the central part of the gland. Neither duct growth nor alveoli are observed. The mammary glands of the non hyp. ect. partners present, in all but 3 experiments with a limited response, proliferated ducts widely distended with secretion as in the foregoing experiments of table 10 and 11.

The 14 experiments included in table 13 are performed on 14 parabiotic rats in the same way as those of table 12 except that the dose of estradiol benzoate injected into the non hyp. ect. castrate is increased tenfold. The mammary glands are examined at different intervals from 10 to 23 days after the first injection of progesterone (expts. 2—6 and 10—12 are a continuation of those of table 11; expts. 1, 13, 14 of expts. of table 12, and expts. 7, 8 of expts. of table 9 a). The mammary glands of the hyp. ect. partners show a development of the duct system, ducts covered with side buds and in seven cases true acini (fig. 12 and 13). The microscopic picture of the glands with acini is similar to that of a mammary gland of a normal rat during the second half of pregnancy. — The ovaries present are atrophic. — The mammary glands of the non hyp. ect. castrated partners are similar to those described for the experiments of table 11.

#### Group D: Comments.

The changes observed on the mammary glands of the castrated partner are similar to those demonstrated by other investigators in estrogen treated single animals with intact hypophysis.

The well established inhibitory action exerted by estrogens on the anterior pituitary with respect to the output of gonadotrophic hormones is indicated by the absence of the ovarian hypertrophy occurring, as described for groups A and B, when a female rat is joined in parabiosis to an untreated castrate. The dose regularly preventing ovarian hypertrophy is found to be 100  $\mu\text{g}$  given every other day. BIDDULPH et al, 1940, found the dose of estradiol preventing the ovarian hypertrophy in a non hyp. ect. female rat united in parabiosis to a castrated one to be considerably lower (0,025  $\mu\text{g}$  per day). However, the duration of these authors experiments is only 11 days. The estradiol treatment is begun immediately after parabiosis and castration. The output of gonadotrophic hormones by the castration hypophysis which is increasing during the first 2 weeks after castration (EVANS and SIMPSON, 1929) can hardly have reached its highest level during that short period. CUTULY & CUTULY (1938) working with male rats found the dose of estradiol preventing the hypertrophy of the testis in the hyp. ect. co twin of a castrate to be about 100  $\mu\text{g}$ . Thus my observations on female parabiotic rats agree well with those of CUTULY made on males.

The observations made on the mammary glands of the untreated hyp. ect. co twins of table 11 point to a stimulating action of estradiol benzoate when injected into the partner with an intact hypophysis. For reasons mentioned in the introduction it may be precarious to draw conclusions from observations on single mammary glands if the changes are small. However, the conclusion that estrogen, injected into the partner with intact hypophysis exerts a stimulating effect is supported by the observations from experiments summarized in table 13.

Progesterone was given to the hyp. ect. partner (table 12 and 13) in order to make the effect exerted by the estrogen, given to the non hyp. ect. castrated co twin more conspicuous. It is well established that progesterone alone in a dose of 5 mg, as used in the present investigation, has no effect on the mammary gland of the hyp. ect. rat, not even if the anterior pituitary is left intact (e. g. ASTWOOD & GESCHICKTER, 1938, SELYE, 1940). That the dose of

5 mg progesterone does not exert a direct effect on the mammary gland when given alone is supported by the experiments of tables 8, 9 and 12.

Thus estrogen administered to the partner with an intact hypophysis in amounts inhibiting the output of gonadotrophic hormones produces proliferative changes in the mammary gland of the hyp. ect. co twin and makes the parenchyma responsive to subthreshold doses of progesterone.

## Discussion and conclusions.

The conclusive observations made in the present investigation (illustrated by fig. 14, I to IV) are:

I: Group A: When a normal female rat (= recipient) is joined in parabiosis to a castrated littermate female rat (= donor) the ovaries and mammary glands of the recipient become hypertrophic. The mammary gland of the donor rat remains undeveloped.

II: Group B: When a hyp. ect. female rat (= recipient) is joined in parabiosis to a castrated mate (= donor) the mammary glands of both the animals remain undeveloped inspite of the presence of hypertrophic ovaries in the hyp. ect. recipient.

III: Group C: After injection of high doses (100  $\mu$ g) of estradiol benzoate into the hyp. ect. co twin of a castrated partner with intact hypophysis the mammary gland of the hyp. ect. parabiont remains undeveloped. Progesterone given to the hyp. ect. partner does not exert a local action on the mammary gland of the hyp. ect. parabiont.

IV: Group D: Injection of high doses of estradiol benzoate (100  $\mu$ g) into the non hyp. ect. castrated co twin (= donor) of a hyp. ect. partner (= recipient) causes proliferative changes in the mammary gland of the recipient and makes it responsive to subthreshold doses of progesterone. The mammary gland of the donor rat shows proliferated ducts widely distended with secretion.

How can the observations made in the present investigation be explained with regard to the theories put forward by other investigators on the hormonal control of mammary gland growth?

1) Do the ovarian hormones exert their action directly on the mammary gland? — Should the unresponsiveness of the mammary gland, which was demonstrated by many investigators in single hyp. ect. animals be ascribed to the general adverse effects of hypophysectomy?

If this assumption is true the reaction of the mammary gland of the hyp. ect. parabiont of group B and C should be stronger than that of group D (fig. 14, II, III and IV), because the amount of ovarian hormones acting in the hyp. ect. animal is far greater in group B and C than in group D. My experiments, however, prove the contrary to be true.

Further the amount of estrogens administered to the hyp. ect. partners in group C is more than 10 times larger than that estimated to be released by the hypertrophic ovaries in group B. If the effect of the ovarian hormones was direct it should be possible to demonstrate a relationship between dosage and response as was demonstrated in animals with intact hypophysis treated with estrogens (e. g. ASTWOOD et al, 1937). But the examination of the mammary glands of the hyp. ect. partners of group B and C does not reveal a dose-response relation of that kind. With the exception of the experiments of tables 9 and 9 a, which will be considered later, the growth is found to be roughly of the same order in the experiments of tables 4 to 8, regardless of the dose of estrogen acting upon the mammary gland. — Further the positive reaction of the mammary gland of the hyp. ect. partner studied under the conditions of group D makes it very doubtful that the negative result obtained in groups B and C could be caused by a general adverse effect of the hypophysectomy.

2) Do the ovarian hormones stimulate mammary growth indirectly by releasing the anterior pituitary hormones, Mammogen I and II, which in turn exert their trophic action on the mammary gland (TURNER and his school)?

The hormones, Mammogen I and II, believed by MIXNER and TURNER to be proteins related to the known anterior pituitary hormones would be expected to pass from one parabiont to the other

according to the same rules as those established for anterior lobe hormones. In group A the mammary gland of the castrated non hyp. ect. co twin of a non hyp. ect. partner with hypertrophic ovaries and mammary glands would be expected to proliferate extensively. But this is not the case. In group D the high doses of estradiol benzoate given to the parabiont with intact hypophysis cause a very strong effect on the mammary gland of the injected partner, whereas the reaction of the mammary gland of the hyp. ect. co twin is only slight (table 10 and 11). A mammogenic factor released in excess from the anterior pituitary would be expected to cross into the hyp. ect. partner in amounts sufficient to produce a hypertrophy similar to that occurring in the injected co twin.

The theory as put forward by TURNER and his school that the ovarian hormones exert their specific action via the hypophysis only, is not supported by the findings made in the present investigation. But the assumption adhered to also by other workers (e. g. SELYE & COLLIP, 1936, LAQUEUR & FLUHMANN, 1942) that steroid hormones can release pituitary factors with mammogenic properties should be kept in mind. This assumption is supported by observations on the mammary gland and the anterior pituitary of rats and rabbits treated with estrogens after cutting the pituitary stalk (JACOBSON, 1947). In these experiments on stalk cut rabbits and rats, which are later to be published in extenso, I found a proliferation of ducts widely distended with secretion following estrogen treatment. The reaction of the mammary gland occurred simultaneously with changes in the microscopic picture of the anterior pituitary (WESTMAN & JACOBSON, 1942).

3) Do the ovarian hormones act directly on the mammary gland synergistically with anterior pituitary factors?

The observations made on group A, B and C, favour this view. The synergistic pituitary factor might be present in the castrated, non hyp. ect. co twin of the partner with the hypertrophic ovaries (group A and B) or of the castrated non hyp. ect. co twin of the hyp. ect. partner injected with estradiol benzoate (group C<sub>1</sub>), but the amount of ovarian hormones crossing into the castrated, non injected parabiont is insufficient for stimulating the mammary glands to additional growth. The absence or scarcity of growth processes in

the mammary gland of the hyp. ect. parabiont inspite of the presence of ovarian hormones in amounts sufficient to stimulate abundant mammary gland growth in animals with intact hypophysis (group B and C) might be explained by the same assumption. It is well established that anterior pituitary hormones exert their action in the recipient of a pair of parabiotic twins only if the output from the hypophysis of the donor rat is increased (DU SHANE et al, 1935, WESTMAN & JACOBSON, 1944). It seems reasonable to conclude that the same should hold true for the synergistic anterior pituitary factor, and that the failure to induce mammary gland growth in the hyp. ect. parabiont in group B and C is due to a lack of the synergistic factor.

But in order to find an interpretation of the observations made in the experiments of group D (table 11 and 13) and C<sub>2</sub> (table 9 and 9 a) the possibility mentioned above that the ovarian hormones are capable of releasing a pituitary factor with mammogenic properties must be taken into account.

What is the difference between the experiments in which no or only a very slight growth response of the mammary gland of the hyp. ect. parabiont could be obtained (group B and C) and those in which a more or less extensive development was promoted in the mammary gland of the hyp. ect. parabiont (group D)?

In the experiments of group B and C the ovarian hormones exert their action solely or chiefly in the hyp. ect. parabiont. The amount of estrogens passing into the non hyp. ect. co twin is too small to exert an action on effector organs with a higher threshold, e. g. the anterior pituitary.

In group D the action of estradiol is exerted chiefly on the parabiont with intact hypophysis. Otherwise the conditions are the same as for group B and C. The doses of estradiol benzoate are high enough to exert an action on the hypophysis. The output of gonadotrophic hormones is inhibited. Granted that this effect on the anterior pituitary, which is dependant on the dosage, involves the stimulation of the output of hormones with mammogenic properties, which in turn enable the ovarian hormones to exert their direct action on the mammary gland, the observations not understandable by the assumptions discussed hitherto can be explained as follows:



Group D, table 11 and 13; 100  $\mu$ g estradiol benzoate, injected into the partner with intact hypophysis, cause proliferative processes on the mammary gland of the hyp. ect. co twin. The proliferation is increased when in addition progesterone is injected into the hyp. ect. parabiont (fig. 14, IV).

The estradiol benzoate stimulates the output of anterior lobe factors with mammogenic properties to a level sufficient to elicit an effect on the mammary gland of the hyp. ect. parabiont. The amount of estrogen crossing into the hyp. ect. co twin of the injected partner is small, and therefore the direct action only slight. The direct stimulus given in addition by the progesterone increases the response considerably.

The experiments of group D table 10 and 12 are performed in the same way as those just discussed, but with one tenth of the dose of estradiol benzoate. The 10  $\mu$ g dose is too small with regard to the amount passing into the non injected co. twin and possibly as to the action on the anterior pituitary. The response of the mammary gland of the hyp. ect. parabiont is very limited or absent.

In the experiments of group C<sub>2</sub> table 9 and 9a the slight reaction of the mammary glands of both partners points to a small amount of estrogen and a greater amount of pituitary hormones acting on the mammary gland of the partner with intact hypophysis whereas the converse conditions are present in the hyp. ect. partner.

From the considerations put forward I conclude that the ovarian hormones exert their action on the mammary gland directly as well as indirectly via the anterior pituitary. This view agrees with the observations of previous workers 1) that mammary gland development can be induced by anterior pituitary extracts in the absence of the gonads and the adrenals and the hypophysis (GREEP & STAVELY, 1941, REECE & LEONARD, 1941, COWIE & FOLLEY, 1944, 1947) and 2) that in animals with intact hypophysis proliferation of a mammary gland can be promoted by a percutaneous application of estrogen on that gland while the untreated adjacent glands remain undeveloped (e. g. SPEERT, 1940).

## Summary.

The mode of action of estrogens on the mammary gland is studied in parabiotic rats.

Evidence is presented in favour of the view that the ovarian hormones stimulate mammary gland growth by acting directly on the mammary gland and indirectly by releasing an anterior pituitary factor which in turn stimulates mammary gland growth synergistically with the ovarian hormones.

The main findings supporting this view are:

1) Mammary gland growth does not occur in the hyp. ect. female rat united in parabiosis with a non hyp. ect. castrate in spite of the presence of hypertrophic ovaries.

2) No dose-response relationship is found when the hyp. ect. co twin of a partner with intact hypophysis is injected with increasing doses of estradiol benzoate. The mammary glands of both partners remain more or less undeveloped.

3) When a parabiont with intact hypophysis is injected with estradiol benzoate proliferative changes occur in the mammary gland of the hyp. ect. co twin. The proliferation is considerably increased by administration of a subthreshold dose of progesterone to the hyp. ect. parabiont.

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## TABLES.



TABLE 1.

A castrated rat is united with a normal mate.

Exp.	No. of the pair	Castr. days before parab.	Days after parab.	Ovary mg	M a m m a r y g l a n d o f			
					non castr. partner		castr. partner	
					B	A S	B	A S
1	23	14	11	58 54	2	3 1	1	— —
2	39	18	11	128 112	3	3 1	2	— —
3	38	17	13		3	3 1	2	— —
4	35	0	14		3	3 1	2	— —
5	21	13	15	50 51	3	3 1	2	— —
6	36	10	17		3	3 3	—	— —
7	30	15	20		3	3 2	2	— —
8	41	8	20		3	3 2	1	— —
9	48	0	37		3	3 2	2	— —
10	50	0	37		3	3 2	1	— —
11	50	0	61		3	3 2	2	1 —
12	48	0	62		3	3 1	2 <sub>c</sub>	— —
13	50	0	67	400 290	3	3 2	2 <sub>c</sub>	— —
14	48	0	87	60 78	3	3 2	2	1 1
					3	3 3	1	— —

As the glands were studied on frozen sections and/or paraffin sections only dates conc. side buds, acini and secretion are given.

Abbreviations see page 7.



TABLE 2.

Two normal rats 20 to 28 days old are united in parabiosis.  
After parabiosis one partner is castrated.

Exp.	No. of the pair	Days after castr.	Ovary mg	M a m m a r y g l a n d o f											
				non castrated partner					castrated partner						
				area	D	B	E	A S	area	D	B	E	A S		
1	285	21	10	3,5	2	1	2	0	0						
2	235	21	35	5	2	2	0	0	0	4	1	2	0	0	0
3	234	21	32	5	2	2	0	0	3	4	2	2	0	0	0
4	226	33	50	4	1	2	0	2	3	5	2	2	0	0	0
5	224	33	45	6	2	3	0	3	3	5	2	2	0	0	0
6	269	39	37	3	1	2	0	0	3	4	2	1	2	0	0
7	223	40	95	10	3	3	0	3	2	5	2	2	0	0	0
8	221	40	47	5	2	2	0	0	3	5	2	2	0	0	0
9	234	40	c:a 70	8	2	2	0	0	3	4	1	1	0	0	0
10	235	53	75*)	8	3	3	0	0	3	5	2	1	0	0	0

\*) The animal is half castrated.

Abbreviations see page 7.

TABLE 3.  
Two normal rats 21 days old are united in parabiosis. A fortnight after parabiosis one partner is castrated and the other sham hyp. ect.

Exp.	No. of the pair	Days after sham hyp. ect. and castr.	Ovary mg	M a m m a r y g l a n d o f											
				sham hyp. ect. partner						castrated partner					
				area	D	B	E	A	S	area	D	B	E	A	S
1	152	10	—	2	1	3	1	0	0	2	1	1	2	0	0
2	334	12	> 40	2,5	1	2	0	0	2	2	1	1	1	0	0
3	246	14	20	2,5	1	3	0	0	0	3	1	1	2	0	0
4	261	14	—	3	1	2	0	0	0	2	1	1	2	0	0
5	255	15	c:a 50	3,5	2	3	0	0	0	4	2	0	2	0	0
6	257	15	> 40	3	2	2	2	0	0	3	1	1	2	0	0
7	262	20	33	3,5	2	3	0	1	2	3,5	2	2	0	0	0
8	263	20	21	4	1	1	2	0	0	4	1	1	2	0	0
9	265	20	43	3	2	3	1	0	0	5	3	1	2	0	0
10	316	22	40	2	1	3	0	0	0	2,5	1	1	1	0	0
11	331	26	165	5	1	3	0	3	0	4	2	1	2	0	0
12	246	28	> 100	3	1	3	0	3	0	3,5	2	2	0	0	0
13	350	29	30	4	1	3	0	3	0	4	2	1	0	0	0
14	255	30	51	2,5	1	3	0	3	0	3	2	1	1	0	0
15	257	30	86	3	1	3	0	0	0	3	2	1	0	0	0
16	261	31	32	2,5	1	3	0	0	0	3,5	1	1	1	0	0
17	275	32	44	4	2	3	0	3	0	3,5	2	1	2	0	0
18	327	32	44	3	2	2	0	0	0	3	2	1	0	0	0
19	262	39	115	3	1	3	0	3	1	3	2	1	2	0	0
20	263	39	72	4	2	3	0	1	0	3	1	1	0	0	0
21	265	39	245	4	2	3	0	1	1	4	3	1	0	0	0
22	246	40	113	4	2	3	0	3	0	4	2	1	0	0	0
23	261	61	167	6	2	3	0	3	1	7	3	1	2	0	0

Abbreviations see page 7.

TABLE 4.

Two normal rats are united in parabiosis. About a fortnight after parabiosis one partner is castrated and the other hypophysectomized.

Exp.	No. of the pair	Age at parab. days	Days after hyp. ect. and castr.	Ovary mg	M a m m a r y g l a n d o f											
					hyp. ect. partner				castr. partner				area			
					area	D	B	E	A	S	area	D	B	E	A	S
1	162	24	5	—	1,5	1	1	1	0	0						
2	173	21	6	14	1,5	1	1	2	0	0						
3	172	21	6	18	2	1	1	1	2	0						
4	169	33	8	11	2	1	1	0	0	0						
5	127	40	10	20	1,5	1	1	2	0	0						
6	154	22	10	—	3	1	1	2	0	0						
7	123	48	10	22	3	1	1	2	0	0						
8	244	23	14	26	2	1	1	2	0	0						
9	256	22	15	40	2,5	1	1	2	0	0						
10	116	42	15	45	1,5	1	1	2	0	0						
11	238	22	16	22	3	1	1	1	0	0						
12	253	21	21	—	3	1	1	1	0	0						
13	369	22	21	40	2	1	1	2	0	0						
14	267	22	22	14	3	1	1	2	0	0						
15	318	25	22	> 40	1,5	1	1	2	0	0						
16	238	22	27	100	2	1	1	2	0	0						
17	244	23	28	100	2	1	1	2	0	0						
18	345	24	29	75	2,5	1	1	2	0	0						
19	256	22	30	48	2,5	1	1	2	0	0						
20	298	37	32	70	4	1	1	2	0	0						
21	244	23	40	117*)	3	1	1	2	0	0						
22	238	22	40	159*)	2,5	1	1	2	0	0						
23	253	21	41	41	2,5	1	1	2	0	0						
24	267	22	41	60*)	3	1	1	1	0	0						
25	253	21	61	226*)	3	1	1	1	0	0						
26	267	22	61	134*)	4	1	1	0	2	0						
27	256	22	62	118*)	3	1	1	1	0	0						

\*) The animal is half castrated.

Abbreviations see page 7.

TABLE 5.

A rat, castrated about 35 days old, is united in parabiosis with a normal rat. Hypophysectomy is performed in the latter after hypertrophy of the ovaries and mammary glands is established.

Exp.	No. of the pair	Parab. after castr. days	Hyp. ect. after parab. days	Days after hyp. ect.	Ovary mg	M a m m a r y g l a n d o f			
						hyp. ect. partner		castrated partner	
						B	A S	B	A S
1	29	16	19	2	—	3	3 0		
2	30	15	20	6	—	3	2 0	1	0 0
3	37	17	13	14	105	2	1 0	2	0 0
4	38	17	13	14	105	2	0 0	1	0 0
5	41	8	20	14	144	2	0 0	1	0 0
6	58	7	14	16	75	2	0 0	2	0 0
7	53	3	18	16	28	2	0 0	2	0 0
8	26	13	12	17	—	2	0 0	1	0 0
9	29	16	19	29	—	2	0 0	2	0 0
10	30	15	20	29	200	2	0 0	2	0 0
11	29	16	19	43	202 222	2	0 0	2	0 0
12	35	0	14	51	217	2	0 0	2	0 0
13	42	8	20	73	320 272	2	0 0	1	0 0

As the glands were studied on frozen and paraffin sections only dates conc. side buds, acini and secretion are given. Abbreviations see page 7.

TABLE 6.  
A non hyp. ect. castrated rat is joined in parabiosis with a hyp. ect. rat. The hyp. ect. partner is injected with 10  $\mu$ g estradiol benzoate every other day.

Exp.	No. of the pair	First inj.		Days after first inj.	Ovary mg	M a m m a r y g l a n d o f													
		castr.	days after			hyp. ect. partner					castr. partner								
						area	D	B	E	A	S	area	D	B	E	A	S		
1	53	37	16	14*)	—	—	1	2	2	0	0	—	2	1	2	0	0		
2	58	37	16	14*)	—	—	1	2	0	0	0	—	2	1	1	0	0		
3	128	11	11	14	24	4	1	1	2	0	0	4	2	1	2	0	0		
4	162	5	5	14	6	1,5	1	1	2	0	0	2	1	1	1	0	0		
5	73	0	6	14*)	—	—	1	2	1	0	0	—	1	2	2	0	0		
6	154	10	10	18	32	2,5	1	1	1	0	0	3	1	2	2	0	0		
7	169	8	8	18	47	3	1	1	2	0	0	4	2	2	2	0	0		
8	53	37	16	28*)	> 40	—	—	2	—	0	0	—	2	1	1	0	0		
9	58	37	16	28*)	102	—	—	1	0	0	0	—	1	2	0	0	0		
10	73	0	6	32*)	66	—	1	1	1	0	0	—	2	2	0	0	0		

\*) 10  $\mu$ g estradiol benzoate in 0.1 ml olive oil (cf page 6).  
Abbreviations see page 7.

TABLE 7.  
The experiments of table 6 continued with 100  $\mu$ g estradiol benzoate every other day.

Exp.	No. of the pair	Days after first inj. of 100 $\mu$ g	Ovary mg	M a m m a r y g l a n d o f											
				hyp. ect. partner					castr. partner						
				area	D	B	E	A	S	area	D	B	E	A	S
1	58	6*)	castr.	—	1	3	0	0	0	—	2	1	1	0	0
2	53	13*)	77	—	1	2	1	0	0	—	2	2	1	0	0
3	128	14	17	2,5	1	1	2	0	0	5	2	2	1	0	0
4	169	14	castr.	2,5	1	1	2	0	0	5	2	2	1	0	0
5	154	15	41	4	2	1	2	0	0	6	2	2	1	0	0
6	162	15	8	3	2	1	2	0	0	5	3	2	0	0	0

\*) 100  $\mu$ g estradiol benzoate in 0,1 ml olive oil (cf page 6).

Abbreviations see page 7.

TABLE 8.

A non hyp. ect. castrated rat is united in parabiosis to a hyp. ect. castrated rat. The hyp. ect. partner is injected with 10  $\mu$ g estradiol benzoate and 5 mg cryst. progesterone on alternate days.

Exp.	No. of the pair	Days after first inj. of prog.	M a m m a r y   g l a n d   o f														
			hyp. ect. partner					castr. partner									
			area					area									
			D	B	E	A	S	D	B	E	A	S	D	B	E	A	S
1	298	13	4	1	2	1	0	0	3	2	2	1	0	0			
2	73*)	17	3	1	2	0	0	0	4,5	2	2	0	0	0			
3	345	18	3	1	2	1	0	0	3,5	2	1	0	0	0			
4	354	18	3	1	2	0	0	0	2	1	2	2	0	0			
5	362	18	3	1	2	0	0	0	3	1	2	0	0	0			
6	332	19	4	1	2	1	0	0	5	2	2	0	0	0			
7	328	22	—	1	1	0	0	0	—	2	1	0	0	0			

\*) Half castrated; 10  $\mu$ g estradiol benzoate in 0.1 ml olive oil (cf page 6).

Abbreviations see page 7.

TABLE 9.

The experiments are performed as those of table 8 but the dose of estradiol benzoate is increased to 100  $\mu$ g. Experiments on incompletely hyp. ect. animals are included.

Experiments on incompletely hyp. test.

Exp.	No. of the pair	Days after first inj. of prog.	M a m m a r y g l a n d o f												Remnant of ant. lobe
			hyp. ect. partner						castrated partner						
			area	D	B	E	A	S	area	D	B	E	A	S	
1	128	14	5	2	2	2	0	0	5	2	2	1	0	0	0
2	73 <sup>a)</sup>	14	—	1	2	0	0	0	—	2	3	0	—	1	0
3	169	16	2, <sup>6</sup>	1	1	1	0	0	5	2	1	1	0	0	0
4	154	17	5	2	2	0	0	0	6	3	2	0	—	2	0
5	162	17	4, <sup>5</sup>	1	2	0	0	0	7	3	3	0	0	2	0
6	168	16	3	2	1	1	0	0	6	3	2	0	0	0	(+)
7	163	17	3, <sup>6</sup>	1	2	0	0	0	6	2	2	1	0	2	(+)
8	170	20	2	1	2	1	0	0	7	3	2	1	—	1	(+)
9	171	20	—	1	2	0	2	0	—	2	2	0	0	1	-+-
10	161	17	3	1	3	0	3	0	8	3	2	1	0	1	-+-
11	152	17	2, <sup>6</sup>	1	3	0	3	0	8	3	2	0	0	1	-+-
12	95	20	—	1	3	0	3	1	—	1	2	0	0	2	-+-
13	104	32	—	2	3	0	3	2	—	1	2	1	0	2	-+-
14	71	14	—	1	3	0	3	3	—	1	2	0	0	0	-+-

<sup>a)</sup> Half castrated. The weight of the ovary is 307 mg at the end of the expt.; 100  $\mu$ g estradiol benzoate given in 0,1 ml.

(+) = small number of anterior lobe cells.

-+- = larger remnant of anterior pituitary.

+++ = large remnant of anterior pituitary.

Abbreviations see page 7.



TABLE 9 a.

A hyp. ect. rat is united to a non hyp. ect. castrate. The hyp. ect. parabiont is injected with 5 mg. cryst. progesterone every other day. Experiments on incompletely hyp. ect. animals are included.

Exp.	No. of the pair	First inj. days after castr. hyp. ect.	Days after first inj.	Ovary mg	M a m m a r y g l a n d o f										Remnant of ant. lobe		
					hyp. ect. partner					castrated partner							
					area	D	B	E	A	S	area	D	B	E		A	S
1	298	33	14	144	3	1	2	0	0	—	—	3	2	0	0	0	0
2	212	16	15	34	2,5	1	2	1	0	0	2	1	2 <sub>c</sub>	2	0	0	0
3	318	22	16	50	3	1	2	1	0	0	5	2	2	0	0	—	0
4	318	57	16	400	4	1	3 <sub>c</sub>	0	0	1	6	3	2	0	0	—	0
5	364	30	17	348 346	3	1	2 <sub>c</sub>	0	0	0	4	2	2	0	0	0	0
6	367	30	17	290 253	3,5	1	2 <sub>c</sub>	0	0	0	3,5	2	2	0	0	0	0
7	332	31	18	445	1	3 <sub>c</sub>	0	0	0	0	4	2	2	0	0	0	0
8	328	36	19	90	4	1	2 <sub>c</sub>	0	0	0	4	2	2	0	0	0	0
9	205	27	15	31	—	1	3	0	0	3	—	2	1	0	0	0	++
10	209	16	15	140	2,5	2	3	1	0	1	4	2	2	2	0	0	+
11	210	16	15	120	4,5	2	3	0	3	0	5	2	1	2	0	0	+
12	316	57	16	157	4	1	2	0	2	0	4	2	2	0	0	0	++
13	327	36	19	124	3	1	3	0	3	0	3	2	1	1	0	0	++

+- = small remnant of anterior pituitary.

++ = larger remnant of anterior pituitary.

Abbreviations see page 7.

TABLE 10.

A non hyp. ect. castrated rat is united in parabiosis with a hyp. ect. rat. The non hyp. ect. partner is injected with 10  $\mu$ g estradiol benzoate every other day.

Exp.	No. of the pair	First inj.		Days after first inj.	Ovary mg	M a m m a r y g l a n d o f											
		castr.	hyp. ect.			hyp. ect. partner						castr. partner					
						area	D	B	E	A	S	area	D	B	E	A	S
1	101	0	8	14	9	3	1	1	0	0	0	—	2	3	0	0	3
2	103	0	8	14	—	2	1	1	0	0	0	4	2	2	0	0	3
3	111	0	8	15	—	2	1	1	2	0	0	5	2	3	0	0	3
4	92	0	11	15	5	4	1	2 <sub>c</sub>	2	0	0	4	2	3	0	0	3
5	93	0	11	15	—	2	1	1	0	0	0	4	1	3	0	0	3
6	116	16	16	14	50*)	4	1	2	0	0	0	6	2	2	0	0	2
7	126	10	10	16	8*)	4	2	1	0	0	0	5	2	3	0	0	3
8	127	10	10	16	8*)	4	2	2	0	0	0	4	2	2	0	0	3
9	172	6	6	17	21*)	3	1	1	1	0	0	4,5	2	3	0	0	3

\*) The animal is bald

\*) The animal is half castrated.  
Abbreviations see page 7.

TABLE 11.  
The experiments of table 10 continued with estradiol benzoate 100  $\mu$ g every other day.

Exp.	No. of the pair	Days after first inj. of 100 $\mu$ g	Ovary mg	M a m m a r y g l a n d o f											
				hyp. ect. partner					castr. partner						
				area	D	B	E	A	S	area	D	B	E	A	S
1	103	14	7	5	2	2	0	0	0	4	2	2	0	0	3
2	111	15	4	5	1	1	2	0	0	4	2	2	0	0	3
3	92	19	—	—	1	3	1	0	1	4,5	2	2	0	0	3
4	93	19	5	—	1	2	0	0	0	4	1	2	0	0	3
5	116	14	castr.	—	1	2	0	0	0	—	1	2	0	0	1
6	126	14	castr.	5	2	2	0	0	0	—	1	3	0	0	3
7	127	14	castr.	—	2	2	0	0	0	—	—	2	0	0	3
8	172	16	castr.	4	2	1	1	0	0	6	2	3	0	0	3

Abbreviations see page 7.

TABLE 12.

A non hyp. ect. castrated rat is united in parabiosis to a hyp. ect. castrated rat. The non hyp. ect. partner is injected with 10  $\mu$ g estradiol benzoate every other day. On the alternate day the hyp. ect. co twin is treated with 5 mg cryst. progesterone.

Exp.	No. of the pair	Days after first inj. of prog.	M a m m a r y   g l a n d   o f												
			hyp. ect. partner						castrated partner						
			area	D	B	E	A	S	area	D	B	E	A	S	
1	238	14	3	1	2 <sub>c</sub>	1	0	0	6	2	3	0	0	3	
2	244	14	—	1	1	1	0	0	—	1	2	1	0	2	
3	345	15	3	2	1	1	0	0	3	2	2	0	0	0	
4	354	15	3	1	1	0	0	0	3	2	2	0	0	0	
5	362	18	—	1	2 <sub>c</sub>	0	0	0	—	1	2	0	0	0	
6	238	21	3	1	2 <sub>c</sub>	1	0	0	6	3	3	0	0	3	
7	244	21	4	2	1	1	0	0	6	2	3	0	0	3	
8	256	28	3	1	2 <sub>c</sub>	2	0	0	7	3	3	0	0	3	
9	267	31	3	1	1	0	0	0	5	2	2 <sub>c</sub>	1	0	3	
10	253	33	3,5	2	2 <sub>c</sub>	1	0	0	7,5	3	3	0	0	3	

Abbreviations see page 7.

TABLE 13.

The experiments are performed as those of table 12 but the dose of estradiol benzoate is increased to 100  $\mu$ g.

Exp.	No. of the pair	Days after first inj. of prog.	Ovary*) mg	M a m m a r y g l a n d o f											
				hyp. ect. partner						castr. partner					
				area	D	B	E	A	S	area	D	B	E	A	S
1	362	10		5	2	3	0	0	0	5	2	3	0	0	3
2	92	13	4	4	2	3	0	3	0	3	1	2	0	0	3
3	93	13	4	—	—	3	0	3	0	—	1	2	0	0	3
4	103	14	6	—	2	3	0	3	0	—	1	2	0	0	3
5	111	14	3	4	2	3	0	1	0	4	1	3	0	0	3
6	116	14		5	2	3	2	0	0	—	2	3	0	0	3
7	364	14		5	2	3	0	0	0	7	2	3	0	0	3
8	367	14		4	2	2	0	0	0	5	2	3	0	0	3
9	369	14		5	2	3	0	0	0	5	2	3	0	0	3
10	126	15		5	2	3	0	2	1	6	1	2	0	0	3
11	127	15		5	2	3	0	3	0	5	1	3	0	0	3
12	172	20		5	2	3	0	3	0	7	2	2	0	0	3
13	345	23		5	2	3	1	0	0	6	2	2	0	0	2
14	354	23		8	3	2	2	0	0	7	2	3	0	0	2

\*) One ovary left. All other cases castrated.

Abbreviations see page 7.

## FIGURES.





Fig. 1: Group A, table 3, expt. 17. Part of a mammary gland of a sham hyp. ect. co twin of a castrated mate with intact hypophysis. Development of ducts and acini. Whole mount. Gallocyenin chromalum.  $\times 10$ .



Fig. 2: Group B, table 4, expt. 21. Part of a mammary gland of a hyp. ect. co twin of a castrated mate with intact hypophysis. No development of ducts and acini inspite of the presence of a hypertrophic ovary. End buds present. Whole mount. Gallocyanin chromalum.  $\times 10$ .

Fig. 3: Group B, table 4, expt. 21. Part of a mammary gland of the non hyp. ect. castrated co twin of the hyp. ect. mate, the mammary gland of which is shown in fig. 2. — Same degree of development. Whole mount. Gallocyanin chromalum.  $\times 10$ .

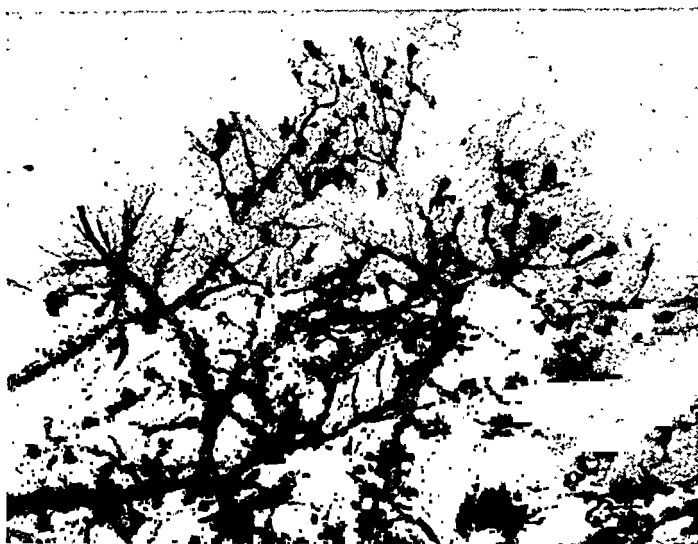


Fig. 2.

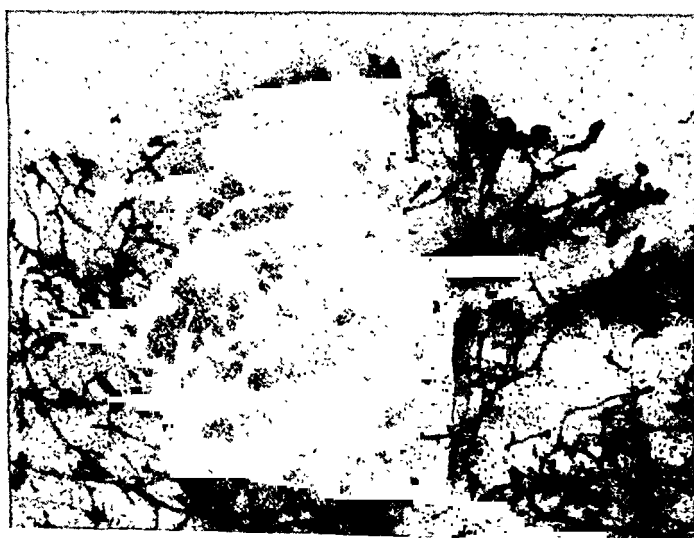


Fig. 3.

Fig. 4: Group A, table 1, expt. 3. Part of a mammary gland of a non hyp. ect. co twin of a castrated mate. — Well developed system of ducts and acini. Frozen section, 300  $\mu$ . Delafield's hematoxylin.  $\times 10$ .

Fig. 5: Group B, table 5, expt. 4. Part of a mammary gland from the same animal as that of fig. 4, but 14 days after hyp. ect. — Involution inspite of persistent hypertrophic ovaries. Frozen section, 300  $\mu$ . Delafield's hematoxylin.  $\times 10$ .

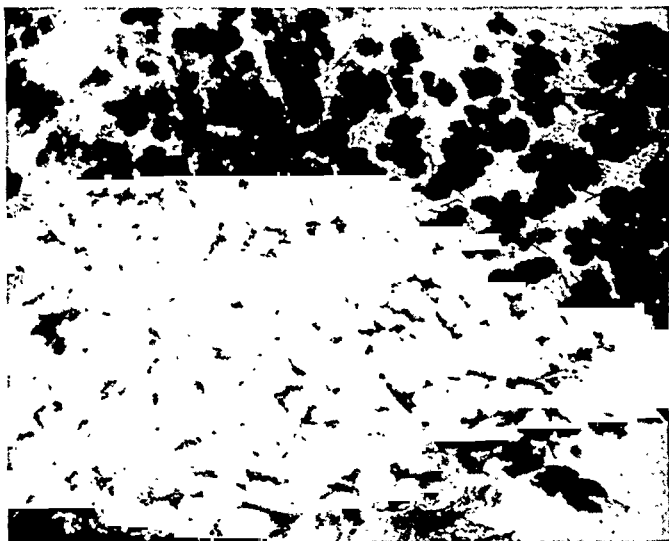


Fig. 4.

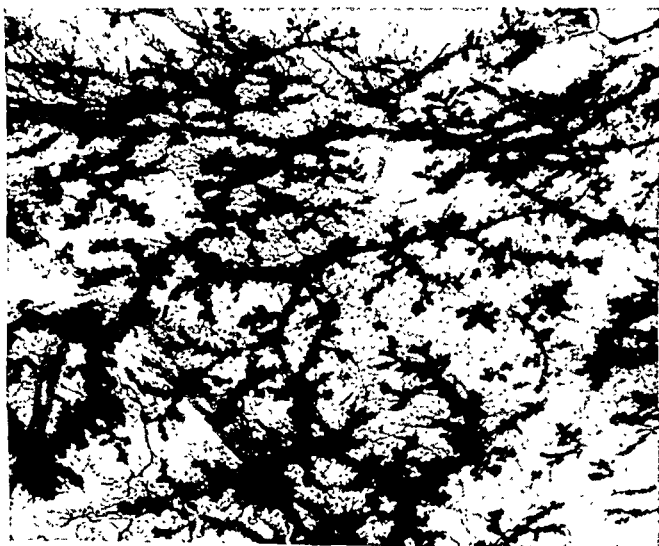


Fig. 5.

Fig. 6: Group B, table 5, expt. 5. Hypertrophic ovary with corpora lutea and large follicles. — 14 days after hyp. ect. The hypertrophy established at hyp. ect. is persistent in the ovary but the mammary gland shows involution. Paraffin section, 5  $\mu$ . Hematoxylin eosin.  $\times 10$ .

Fig. 7: Part of a mammary gland from a single rat 31 days after hyp. ect. Hypophysectomy when the animal was 35 days old. Body weight 60 gms., ovary 2 mg. — Excessive atrophy of the mammary gland. — Whole mount. Gallocyanin chromalum.  $\times 10$ .



Fig. 6.

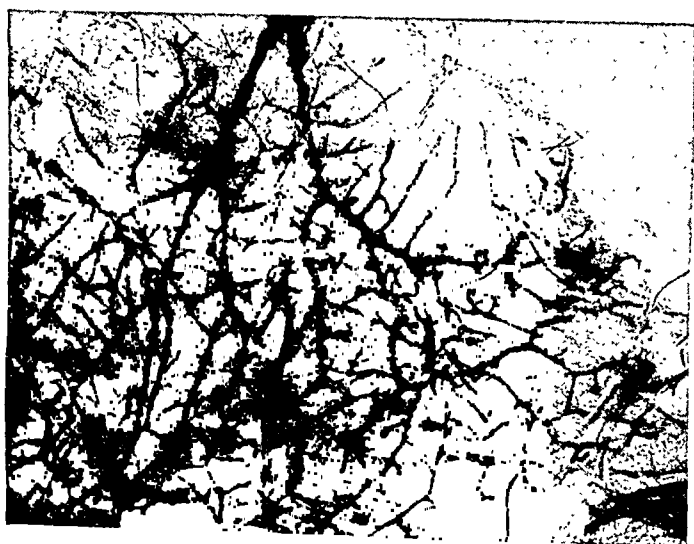


Fig. 7.

Fig. 8: Group C<sub>1</sub>, table 7, expt. 4. Part of a mammary gland from the hyp. ect. co twin of a partner with intact hypophysis. The hyp. ect. parabiont was injected with 10  $\mu$ g and later with 100  $\mu$ g estradiol benzoate. The gland is as undeveloped as that shown in fig. 2. End buds present. Whole mount. Gallocyanin chromalum.  $\times 10$ .

Fig. 9: Group C<sub>2</sub>, table 8, expt. 5. Mammary gland from the hyp. ect. co twin of a partner with intact hypophysis. The hyp. ect. parabiont was injected with 10  $\mu$ g estradiol benzoate + 5 mg cryst. progesterone. — Apart from thickened ducts with a number of side buds present in the central part the gland is atrophic. The mammary gland from the non injected partner shows the same picture. Whole mount. Gallocyanin chromalum.  $\times 10$ .

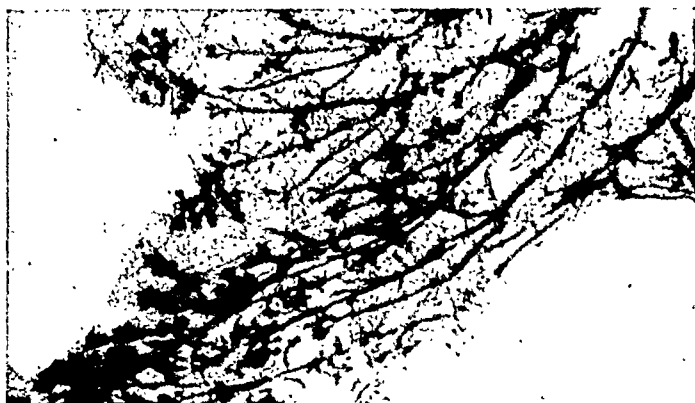


Fig. 8.



Fig. 9.



Fig. 10: Group C<sub>2</sub>, table 9, expt. 5. Part of a mammary gland from the hyp. ect. co twin of a partner with intact hypophysis. The hyp. ect. parabiont was injected with 100  $\mu$ g estradiol benzoate + 5 mg cryst. progesterone. — Proliferation of side buds, evenly distributed over the whole gland. — Whole mount. Galloxyanin chromalum.  $\times 10$ .

Fig. 11: Group D<sub>1</sub>, table 11, expt. 7. Part of a mammary gland from the hyp. ect. co twin of a partner with intact hypophysis, injected with 100  $\mu$ g estradiol benzoate. — Proliferation of side buds evenly distributed over the whole gland. Whole mount. Delafield's hematoxylin.  $\times 10$ .

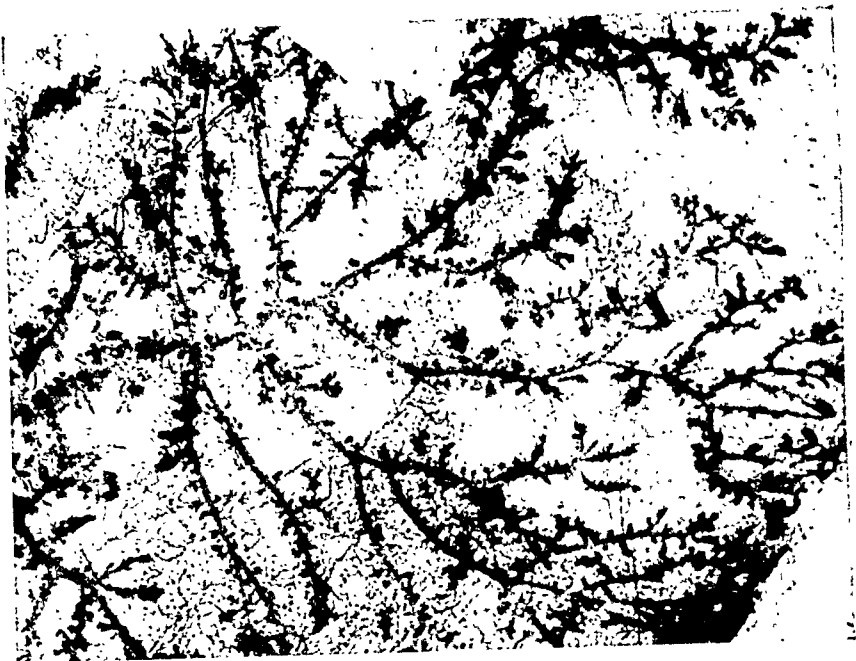


Fig. 10.



Fig. 11.

Fig. 12: Group D<sub>2</sub>, table 13, expt. 11. Part of a mammary gland from the hyp. ect. co twin of a partner with intact hypophysis. The hyp. ect. mate is injected with 5 mg cryst. progesterone, the non hyp. ect. mate with 100  $\mu$ g estradiol benzoate. — Development of ducts and acini. — Whole mount. Gallocyanin chromalum.  $\times 10$ .

Fig. 13: Mammary gland from the hyp. ect. partner of a pair belonging to the same group as that of fig. 12. (No. 93, 20 days after first inj. of progesterone). — Acini. -- Paraffin section, 5 $\mu$ . Hematoxylin eosin.  $\times 145$ .

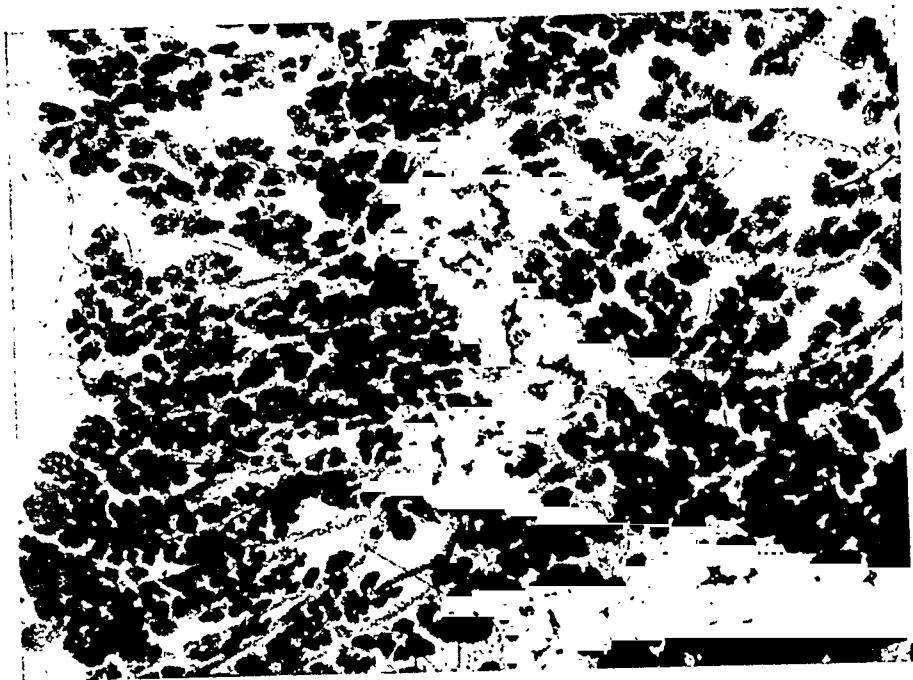


Fig. 12.



Fig. 13.

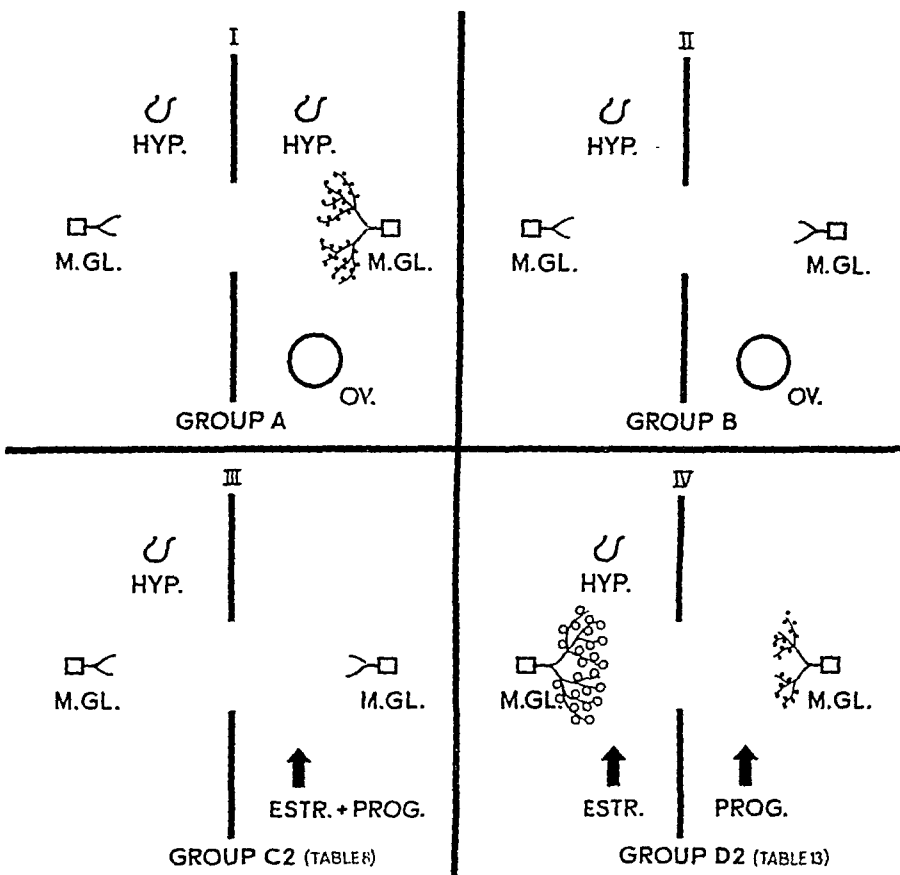


Fig. 14: Scheme illustrating the basic types of experiments elucidating the mode of action of estrogens on the mammary gland.

VARIATIONS IN  
AORTIC PRESSURE IN DOGS  
DURING EXERCISE  
UNDER THE INFLUENCE OF SELECTED DRUGS  
AND BY AORTIC INSUFFICIENCY



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BY

ARNE SKOUBY



*Denne Afhandling er af det lægevidenskabelige Fakultet ved Københavns Universitet antaget til offentlig at forsvares for den medicinske Doktorgrad.*

*København, den 28. Oktober 1948.*

*K. A. JENSEN.*

*h. a. dec.*

## PREFACE

The present investigation was carried out in the Laboratory for the Theory of Gymnastics, University of Copenhagen, during the years 1943—1947.

I am greatly indebted to the Director of the laboratory, Professor *Emanuel Hansen*, Ph. D., for the excellent facilities afforded me at the laboratory. To the Director of the Institute of Neuro-Physiology of Copenhagen, *Fritz Buchthal*, M. D., who stimulated my interest in the problems explored in this paper and followed the work with interest and encouragement, I desire to convey my most cordial thanks. I am also indebted to *Erling Asmussen*, Ph. D., for valuable discussions during the course of the investigation.

I wish to express my best thanks to Miss *Ellen Leffland-Larsen*, who has given me valuable assistance in the experiments and drawn the diagrams, and to Mrs. *Karrebæk-Rasmussen* who also assisted in the experiments and made most of the gas-analyses during the experiments.

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Copenhagen, October 1948.

*Arne Skouby.*



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## INTRODUCTION

Direct measurements of the variations of blood-pressure during the performance of work have not been carried out before. Mean pressure measurements have been undertaken to a limited extent, but without simultaneous and well defined determinations of the quantity of work performed, and the results obtained by different investigators are inconsistent, possibly because the techniques employed have been diverse and in some cases defective. The value of the results obtained by indirect methods has been open to criticism, in that the efficiency of the methods is known only during rest where simultaneous direct and indirect measurements have been possible (*Prytz 1942, Ragan & Bordley 1941*). Our knowledge of the behaviour of the blood-pressure during the performance of work is thus very slender.

The purpose of the present work is to elucidate this problem of how the blood-pressure is adjusted to changing demands, by recording the absolute pressure-fluctuations in the root of the aorta in dogs during the transition period from rest to work, during work in the "steady state" of varying intensity, and in the transition period from work to rest. In addition the pressure variations were recorded in resting dogs under the influence of drugs, and in an unanaesthetised dog with experimentally produced aortic insufficiency; by examining how the pressure fluctuations change under these conditions (where the alterations in the factors regulating the circulation are controlled or partially controlled), and comparing them with the alterations arising during muscular work, it is hoped to localise their cause.

The reason for recording the pressure in the relatively inaccessible root of the aorta instead of in one of the more superficial arteries is that the pressure curve in the beginning of the

aorta, to a higher degree than elsewhere in the arterial system, is an expression of the work done by the heart, in that the modulation to which the primary pressure wave is subjected by wave reflexion and damping in the elastic arterial system is least at this point. As there were available no methods suitable to carry out the proposed investigations, it was first necessary to develop the apparatus and technique, which have been described in a previous paper (*Skouby* 1945), and which, therefore, will only be mentioned briefly in this thesis in the appraisal of the measuring methods used and the technique applied in the experiments.

## CHAPTER I

### PREVIOUS INVESTIGATIONS INTO BLOOD-PRESSURE AND PULSE-RATE DURING EXERCISE

#### A. *Blood-pressure during exercise.*

By measuring the mean pressure in the carotid artery of horses working on a treadmill, *Kaufmann* (1892) found that the pressure as a rule fell and remained below the initial value during the whole working period. As neither the technique nor the results of the experiments are described, it is impossible to make an analysis of them.

This can be done in the experiments of *Zuntz and Hagemann* (1898), again performed on horses working on a treadmill. — The pressure was measured in the transverse facial and carotid arteries and was read on a mercury manometer, or in their later experiments on a large spring manometer, connected to the artery by a tube filled with 25 % magnesium sulphate solution to prevent clotting. For the same reason magnesium sulphate in unspecified quantities was now and then injected into the blood-stream. The working period varied from 1 to 5 minutes and the work performed was very moderate. From a comparison of the mean value of 9 estimations of the resting tension in 6 different animals with the average value of the pressure found in a series of experiments during work, (each of the latter values being the mean of a series of readings during each single experiment), the authors conclude that the mean pressure falls in response to exercise. A review of these experimental results shows that the mean pressures during exercise in 5 of the 8 experiments performed are higher than the resting blood pressure of the animals used; of the remaining 3 experiments, where a fall in pressure was recorded during work, 2 were performed on the same animal.



In experiments on dogs in the same year *Tangl and Zuntz*, using the same technique as in the experiments on horses, but measuring the resting pressure just before exercise began, found that the pressure invariably rose during work and that the rise was greatest at the greatest rate of work. A correlation between the rate of work and the pressure values cannot be established, because the speed of running was only kept constant for very short periods and the "steady state" can scarcely be assumed to have been attained. After the cessation of exercise the pressure falls, descending to the resting value in a few minutes. The authors state that a transient pressure fall is frequently observed at the beginning of exercise; their tables show that such a fall was recorded in 7 out of 17 experiments performed. That it was not particularly pronounced, and perhaps even within the limits of experimental error, is apparent from table I, in which are set out the results of the 7 experiments with details of the mean pressure values measured at rest and again shortly after the beginning of work.

Table I.

Experiment No.	Blood-pressure during rest	Blood-pressure just after the beginning of work
1 .....	115-130 mm Hg	109 mm Hg
2 ..... lying	106-118 "	
standing	121-131 "	112 "
3 .....	126-137 "	118 "
4 .....	111-137 "	126 "
5 .....	113-119 "	98 "
6 .....	103-109 "	96 "
7 .....	103-128 "	99 "

Values for mean pressure before and just after the beginning of muscular work in 7 experiments in which *Tangl & Zuntz* found a transient blood-pressure fall before the rise in mean pressure.

The pressure values quoted are not recorded but read from a manometer. A further uncertainty in this experimental method arises from the fact that the tube connecting the artery with the manometer was filled with a strong solution of magnesium sulphate (which moreover was injected into the blood-

stream), and as the magnesium ion has a pronounced depressant action upon the blood-pressure even in small concentrations (*Haury 1939, Hoff et al. 1939*), its possible influence upon the results cannot be discounted.

In later investigations upon dogs (*Samaan 1935*) in which the pressure in the carotid artery was recorded by means of a mercury manometer, the pressure rose more or less abruptly at the beginning of exercise, reaching a maximum within a few minutes; thereafter it fell and might return to the resting value after 10 minutes exercise. When this did not happen the pressure returned to the resting value immediately after the cessation of exercise. Three hours before the experiments canulae had been inserted in the carotid artery of the dogs under ether anaesthesia; the work was performed by running on a treadmill. Unfortunately in these experiments too the rates of working are not specified and no conclusions can be drawn about the influence of the rate of working upon the pressure rise.

In determinations of the mean pressure in the femoral artery of dogs running on a treadmill *Essex et al. (1939, 1943)* found that it sometimes rises, sometimes remains constant, or else falls during the first minutes of exercise. The further course during work at a constant rate also varied from experiment to experiment; the pressure sometimes rose to a certain level, at which it remained until the cessation of exercise; sometimes it showed a continuous rise throughout the working period, and now and then after a transient fall a new rise supervened, which continued to the end of work; thereafter, in all experiments, the pressure returned to the resting value within a few minutes, uninfluenced by the preceding rate of working. The reasons for these incongruous results may perhaps be sought in the fact that the dogs were running with a canula inserted in one femoral artery, from which a tube led to the mercury manometer recording the pressure; the embarrassing effect of this upon the dogs, together with the vibrations set up during running, may have complicated the recording.

The response to electrically induced muscular work has been investigated in anaesthetised dogs in which the spinal medulla was divided at the 12th dorsal segment. Work was performed

by contractions of the muscles of the hind legs produced by electrical stimulation either directly (*Kramer & Gauer* 1941) or applied to the distal part of the divided medulla (*Euler & Liljestrang* 1946). The results differ from those previously discussed in that the blood-pressure falls as soon as the "work" begins and remains below the resting value so long as the stimulation continues (*Kramer & Gauer*), or rises slowly to the initial level and sometimes, with continuous tetanic contraction, a little over this (*Euler & Liljestrang*). As the pressure falls despite intact function of the pressor-sensitive centres, and as stimulation of the muscles is accompanied as a rule by a rise in blood-pressure if the medulla is preserved from damage, it may be supposed that the injury to the medulla is to blame for the results. As a pressure fall occurs with the cessation of stimulation under these conditions (i. e. when the medulla is not divided) as great as that observed during work in the experiments first quoted, it is probable that other factors are at work (*Kramer & Gauer*). It has previously been shown that stimulation of the posterior roots can bring about a dilatation of the vessels in the corresponding skin area through antidromal conduction in sensory nerves (*Bayliss* 1923). In the experiments under discussion, where the stimulation is applied to the distal part of the divided medulla, it would be difficult to avoid a simultaneous stimulation of the posterior roots by reason of the diffusion of the stimulus, and it is therefore possible that a vaso-dilatation released by this mechanism has influenced the results of the experiments. Since the regulation of the blood-pressure is depressed by narcosis (*Brewer et al.* 1934) it is possible that the chloral or urethane narcosis employed in the experiments has also influenced the results. Further, the fact that the systolic blood-pressure (indirectly measured) in human work experiments is the same whether the work performed is produced by electrical stimulation or in the usual way (*Asmussen et al.* 1943) implies that the pressure fall in the animal experiments is due to their peculiar conditions.

In contrast to the few investigations on the behaviour of the blood-pressure in animals during the performance of work, a very extensive literature deals with the human blood-pressure

during work. For these measurements indirect methods have always been used.

The earliest investigations of this nature were done by *Grebner & Grünbaum* 1899. They employed *Gärtners* Tonometer (1899) for the pressure determinations, and the work, which was dynamic, consisted in the repetition of different exercises in the mechano-therapeutic apparatus constructed by *Herz and Bum* (1899). In all experiments the blood-pressure rose at once after the beginning of work and fell quickly as soon as it ceased. With moderate work of longer duration a constant pressure value was reached, greatest with the greatest output of work, without, however, any more exact correlation between work and rise in pressure being demonstrable. With heavier work the raised pressure varied during the course of work and it was shown that training influenced the results. The fall in pressure after the cessation of work was abrupt in all experiments and the resting values were reached in 5—10 minutes depending upon the preceding output of work.

In succeeding papers it is stated that the blood-pressure during work at a constant rate reaches a maximum in 4—7 minutes and then falls a little in the remainder of the working period (*Bowen* 1904, *Lowsley* 1911, *Gillespie et al.* 1926), whereas in more recent papers it is stated that the indirectly measured systolic pressure reaches a "steady state value" after a few minutes work (*Liljestrand and Zander* 1928, *Eldahl* 1933, *Iljin-Kakujeff* 1937, *Hansen* 1937, *Neukirch* 1938 and *Engels & Niescke* 1942), and that there is a constant relation between blood-pressure level and the rate of work (*Liljestrand & Zander* 1928, *Eldahl* 1933, *Hansen* 1937).

The results obtained by the different investigators for the rises of pressure at various rates of work agree well with one another; the rise of pressure may be as high as 100 mm Hg. The indirectly measured diastolic pressure behaves otherwise, as it rises or falls only a little during work.

Attempts have been made to determine the pressure of freely flowing blood by oscillometric measurements of the pressure value in the cuff which just corresponds to the pressure of the blood on the wall of the artery. The maximal flow pressure by

this method is shown to behave quite differently from the indirectly measured systolic pressure, as it either remains unchanged or only rises a little above the resting value (*Eldahl* 1933, *Hansen* 1937). This circumstance has been explained by the assumption that the values obtained for the indirectly measured systolic pressure during work are considerably above the actual pressure in the brachial artery, due to a "water-hammer" effect when the blood bumps against the compressed artery walls (*Eldahl, Hansen*). The reason that the resting values found by the above methods accord well should lie in the fact that this "water-hammer" effect is small when the velocity of the blood flow is low.

Investigations into blood-pressure relations after the cessation of work are numerous. They show that the systolic pressure reaches the resting value in 5—10 minutes (*Bowen* 1904, *Lowsley* 1911, *Iljin-Kakujeff* 1937, *Neukirch* 1938). Sometimes the pressure level falls to subnormal values, but in these cases it quickly rises to the resting value. No correlation between pressure course and the rate and duration of the preceding work has been demonstrated.

#### B. *Pulse-rate during muscular work.*

Determinations of the pulse-rate in animals have been undertaken in connection with other investigations, involving varying degrees of injury which may possibly have influenced the results (*Samaan* 1935, *Essex et al.* 1939, 1943). The most trustworthy measurements have undoubtedly been made on man, in whom it has been found that the very first pulse-beat after the beginning of work is shortened (*Grünbaum & Amson* 1901, *Buchanan* 1909, *Krogh & Lindhard* 1913).

The pulse-rate rises quickly during the first few seconds and reaches a maximum, after which, in trained persons, it falls to a constant level, while in the untrained it rises throughout the whole working period. The same result is obtained in trained persons when the work is at the limits of their performance. The tempo of working has no apparent influence upon the level attained; only the rate of work influences this and in a comparison between either the units of work or the oxygen intake

per minute and the level reached, there is found an almost linear correlation except for the heaviest work (*Hohwii Christensen* 1931). After the cessation of work the pulse-rate falls quickly at first, then more slowly, and after moderate work the resting level is reached in 2—8 minutes, but after heavier work it takes a longer time. The falling pulse curve shows under these circumstances bigger or smaller waves. A comparison between the fall in pulse-rate and the oxygen intake after the cessation of work shows that the pulse-rate diminishes more slowly than the oxygen intake, especially to begin with (*Lythgoe & Pereira* 1925, *Kagan & Kaplan* 1930).

As mentioned above, pulse determinations are much more trustworthy on man than on animals, but when the changes can be considered to be in principal uniform for both groups, the pulse behaviour if determined simultaneously with other measurements can be used as an indicator of the condition of the animals under observation, which serves particularly to estimate the influence of the preceding operative interference upon the results. When the pulse-rate after a transient fall rises during the remainder of a period of moderate work in *Samaan's* investigations into pulse and blood-pressure behaviour in working dogs, it indicates that the dogs have not been in a natural condition after the preceding operative trauma.

## CHAPTER II

### PREVIOUS INVESTIGATIONS INTO THE COURSE OF THE AORTIC PRESSURE-PULSE CURVE

The earliest investigations relating to the pressure-pulse variations in the aorta were made with inadequate manometers, so that the recorded curves were not a true expression of the pressure course. The work of *Frank* (1905—1913) first led to the construction of manometers sufficiently practicable and trustworthy for a study of the pressure-pulse.

Most investigations have been carried out on anaesthetised animals, often attended by severe surgical interference in gaining access to the measuring site. The first measurements from resting unanaesthetised animals were made by *Gregg et al.* (1937), who used dogs with a previously established carotid loop. Out of consideration for the recordings, which were made with a cistern manometer with a metal membrane (*Hamilton et al.* 1934), the dogs had to be kept immobile during the measurements.

The pressure pulse, which has a characteristic and uniform course for each species (Fig. 1), was first described by *Frank* (1905, 1925, 1927). It is divided into an "ejection" phase (ABCD, Fig. 1), in which both the course and the absolute pressure values are the same as in the left ventricle (*Gregg et al.* 1937), and a diastolic phase corresponding to the period between the closure of the semilunar valves and the beginning of the next systole. The division is marked by a pressure fall supervening quickly upon the end of systole, the incisura DE, which according to *Frank* is due to reflux of blood against the heart after the end of systole. This fall is followed by a rise more abrupt than the fall and 2 or 3 "post-oscillations", c, diminishing in amplitude, supposedly due to oscillations within the elastic

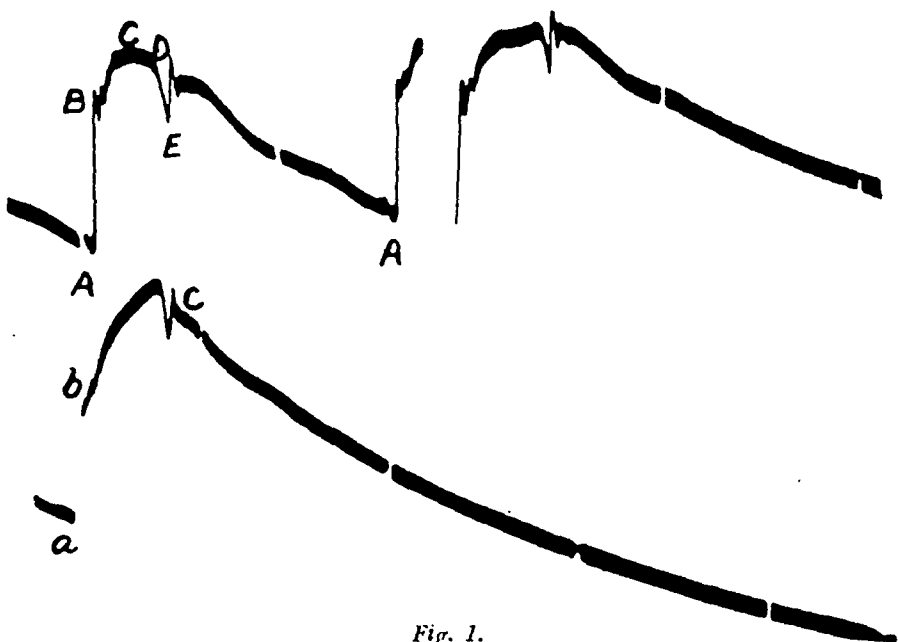


Fig. 1.

Aortic pressure curves (Frank 1905).

AB: Minimum ejection phase.

BC: Maximum ejection phase.

CD: Reduced ejection phase.

DE: Protodiastole.

a: Pre-oscillations.

b: Initial-oscillations.

c: Post-oscillations.

cardio-vascular system itself (Frank 1905). The same appears to be the case with the "pre- and initial oscillations" (a, b) which are of the same frequency (ca. 50 Hz.) and occur immediately before the first steep systolic rise and at its transition into a less steeply rising pressure course (Skouby 1945). In the first part of diastole, after the incisura, there occurs a positive pressure wave of low frequency, which cannot be determined because of its indefinite demarcation. Before the pre-oscillations there occurs inconstantly a pressure wave of considerably longer period than the first, which is ascribed to auricular-systole (Frank 1905, Tigerstedt 1913). The three parts of the "ejection" phase AD are called by Wiggers (1921) "minimum ejection phase", (AB), "maximum ejection phase" (BC), and "reduced ejection phase" (CD). In addition, the incisura (DE) is named protodiastole, because it represents the period from the end of systole to the closure of the semilunar valves. The duration of the individual parts depends upon the pulse-rate. For a pulse-rate



of 75, *Wiggers* states that the ejection-phase in anaesthetised dogs lasts 0,22 second and protodiastole 0,02 second.

In heart-lung preparations it has been shown that the pressure in the left ventricle in the ejection phase rises more quickly, both in the initial steeply rising and in the succeeding less steeply rising parts of the pressure-pulse curve, when the aortic pressure or the venous return increases. A reduced pulse-rate produced by vagal stimulation on the opposite side has the effect of reducing the rate of pressure increase during this period, but the absolute values of the systolic pressure increase nevertheless, due to the longer duration of systole (*Patterson, Piper and Starling* 1914, *Straub* 1926). In anaesthetised animals the aortic pressure follows a course similar to that described for heart-lung preparations except that a slowing of the pulse-rate in such circumstances leads to a greater steepness of the systolic rise (*Wiggers* 1928). This difference in reaction is attributed by *Wiggers* to a reduction of the blood supply to the heart musculature in the heart-lung preparations caused by a low arterial blood-pressure. When the venous return to the heart is diminished the systolic rise decreases in height and steepness both in heart-lung preparations and in intact anaesthetised animals. The course of the diastolic pressure shows in anaesthetised animals a uniform decline. When the volume of circulating blood is diminished, under the influence of either vaso-dilator drugs, shock or bleeding, a pronounced pressure fall occurs in the first part of diastole, so that the course in the later part of diastole may be almost horizontal (*Frank* 1926, *Wiggers* 1928). Entirely parallel changes can be observed during anoxaemia (*Sands & De Graff* 1925). When the heart rate is slowed by stimulation of the vagus fibres to the heart, so that the peripheral resistance and the volume of circulating blood can be considered unchanged, the rate of pressure fall is reduced, but because of the increased duration of diastole the pressure fall may be considerable.

In experimentally produced aortic insufficiency an increased amplitude is invariably found. The pressure variations in the aorta have been investigated by *Wiggers* (1915) in dogs with experimental aortic insufficiency. In these experiments he found

that the first part of the pressure curve was similar to the curve in normal animals with increased diastolic venous inflow, while the rest of the curve corresponded to that in animals with reduced circulating blood volume. In these experiments, however, the pressure course shows a steeper fall at the beginning of diastole as soon as the thorax is opened and before the experimental insufficiency is produced. The increase in amplitude was due to a fall in diastolic pressure and not to a rise in the systolic pressure.

## CHAPTER III

### TECHNIQUE

During the present experiments, including the development of the necessary technique, there were employed 28 dogs, weighing between 8 and 27 kg each.

#### *Training period.*

In this period the dogs were trained to run on the tread-mill used in the work experiments. The tread-mill consists of an iron chassis in each end of which is placed a big roller, the two rollers being 2 m apart. A row of transverse cylinders of lesser diameter and closely spaced is placed between the two big ones, making a firm support for the upper plane part of the running belt, which is a broad driving belt put around the rollers. The two big rollers can be revolved at varying speeds by means of a driving belt from an electro motor. The iron chassis is adjustable so that the inclination of the treadmill may be varied. During training running speeds between 2 and 20 km per hour were used and the inclination was varied between  $4^{\circ}$  and  $16^{\circ}$ . As it was shown during the initial experiments, that the dogs were able to some degree to regulate the speed themselves (by running faster and slower), the speed during later experiments was measured during all recordings — as a rule every other minute — by timing 5—10 revolutions of the belt on a stop-watch. To prevent the dogs jumping off the treadmill a wooden framework 145 cm in length, 50 cm broad and 80 cm high was placed just over the belt. In the training period the dogs were also accustomed to the persons who assisted during the experiments and they were made familiar with metabolism determinations and with the manipulations necessary during the blood-pressure recordings. By these means was

avoided the influence upon the results of increasing training and so far as possible of psychical factors. The training period lasted 1—2 months for each dog, depending upon their receptivity to training.

### *The oxygen intake.*

The oxygen intake was measured in the stationary dog and during running in the "steady state" at different speeds and inclinations of the treadmill. During the measurements the dogs wore an individually fitted cylindrical mask, with walls of rubberised linen and the bottom of fibre plate. In the free margin of the wall is inserted an elastic ring of a circumference gauged to fit tightly around the dog's neck when the mask is in place. In the fibre plate is made a circular opening 2—3 cm in diameter, which is connected by a short rubber tube to a valve of the type used and described by *Enghoff* (1930) in human work experiments. The inspiration side of the valve is in free communication with the atmosphere, but the expiration side is connected by a thick-walled rubber tube to a three-way cock, so that during the experiments the expired air can be led to either of two Douglas bags or the dog can expire to the open air. To prevent air oozing away under the free edge of the mask a stretched rubber bandage 5 cm broad is wrapped several times round that part of the mask fitting the animal's neck. As the mask fits tightly over the occiput and jaw and 4—5 cm of the wall is applied extremely tightly to the hair there is small chance of the air oozing out. It is impossible for the animals to respire when the valve is closed, and while the part of the mask applied to the neck is quite dry at the end of the experiments, the rest is wet from condensed water and saliva.

The mask is made roomy, as smooth running is attained only when the animal can respire with its mouth open. The size of the dead-space, increased on this account, does not influence the results, because only the values of the oxygen intake in the "steady-state" are of interest in the present investigations. After at least 4 minutes running at constant speed, the expired air is collected in 2 or more periods of such duration that 30—40 litres accumulate in each bag. The collecting periods are measured by

a stop-watch, but as the respiration rate is often very high it was not always possible to start and stop the collection in the same respiratory phase. After kneading the bags samples are taken for analysis and the volume of air in the bags measured in a clock spirometer. Analysis of the expired and atmospheric air is carried out in the *Haldane* Gas-Analysis Apparatus as modified by *Krogh*, and from the volume of expired air per minute and the percentages of oxygen and carbon dioxide in this and in the atmospheric air the oxygen intake per minute is determined. The method has been proved to give consistent results for a constant rate of work by repeated measurements on the same animal during a single experiment and during different experiments. The method is only suitable for dogs weighing less than 17 kg, as bigger dogs cannot respire freely in the mask even at low rates of work and after a short time stop running spontaneously, thus rendering an appraisal of the output of work impossible. This also applies to smaller dogs at the higher working rates, so that attempts to carry out working experiments when the oxygen intake exceeds ten times the resting value have been unsuccessful. As the oxygen percentage of the expired air under these circumstances is much diminished, the fact can hardly be due to hyperventilation or to a failing oxygen intake contingent upon the large dead-space, but can most probably be ascribed to the fact that when the resistance to respiration increases, the dogs do not increase their respiratory rates, thereby producing anoxaemia. That the increased resistance should make it impossible to respire satisfactorily seems to be excluded, as the valve used has shown itself well-suited to experiments on man during heavy work.

#### *Recording of the aortic pressure fluctuations.*

1—2 days before the pressure recordings a 10—14 cm long tube made from a Porgès catheter is introduced, under morphine, into the right common carotid artery in such a way that one end lies just at the bifurcation of the brachio-cephalic artery from the aorta, while the other debouches upon the distal part of the front of the neck where it is fixed to the skin. The operation wound is powdered with neutral sulphathiazole and after closure

is dressed with a plaster of Paris collar, through which the outer end of the catheter protrudes. When not in use the catheter is closed by a stylet 6 cm in length whereby the flexibility of the distal part of the catheter is preserved and the risk of lesions of the vessels from violent movements of the neck is minimised. During the measurements the stylet is withdrawn and the catheter is connected by a nut to the end of a glass tube, which contains the condenser manometer in an air-free solution of sodium citrate. The glass tube can be shut off from the catheter by a metal cock and through a side tube communicates with the atmosphere. The condenser manometer is connected by a cable with a high-frequency oscillating circuit, in which capacity variations are converted into voltage variations; the latter are amplified and recorded photographically by means of an oscillograph. For details of the condenser manometer and of the high-frequency and amplifying circuits reference is made to a previously published paper (*Skouby*, 1945). Attention is drawn only to the essential advantage of the system used over that of *Buchthal and Warburg* (1943), that the influence of variations in the capacity of the cable is abolished by inserting a specially constructed transformer between the manometer and the cable. As during working experiments movements of the cable are unavoidable this security is indispensable.

The condition of the experimental animals should so far as possible remain unchanged during the pressure recordings when information upon their behaviour under physiological circumstances is desired. Therefore only those dogs are used in which the general condition after insertion of the catheter was good. Except for a loss of appetite the animals' behaviour was the same during the period of experiment as before the operation. Figure 2 shows a dog 5 days after operation. As the catheter fills up completely the lumen of one carotid artery it must be admitted that the function of the carotid sinus on that side is compromised. However, injury to a single pressure-sensitive area is without significance in the regulation of the circulation when the others are intact (*Heymans* 1929, *Heymans and Bouckaert* 1931). The cerebral blood-supply in dogs is mainly derived from the vertebral arteries and so does not suffer by the



*Fig. 2.*

Dog 5 days after the insertion of the catheter into the right carotid artery. The proximal part of the catheter with indwelling stylet is seen in an aperture in the plaster collar which prevents the dog damaging the catheter and protects the wound.

operative procedure. Experiments have shown that the carotid arteries can be ligated on both sides without the appearance of demonstrable cerebral symptoms.

To permit the carrying on of experiments over a long period it is necessary to prevent the blood in the catheter clotting. For this purpose Dicumarin was used in the initial experiments, but more recently intravenous injection of heparin just before the experimental periods has been the standard method, because the dog not heparinised between experiments does not produce demonstrable embolus when the catheter is correctly placed, i. e.

with the tip lying in the aorta at the bifurcation of the brachiocephalic artery. (The Heparin used was kindly placed at my disposal by the Løvens kemiske Fabrik, Copenhagen). By this means tissue necrosis in the wound is diminished, as the secretion from the incision is greatly reduced by the preservation of the coagulability between experiments. The heparin is given in doses of 8 mg per kg-body weight, which within 2—3 hours after injection reduces the coagulability to such a degree, that no clots are formed in the glass-tube or the catheter for 30—50 minutes after the attachment of the measuring apparatus to the animal. Whether the dog is standing still or running the axis through the glass-tube and catheter remains horizontal, so that no correction for the fluid column between the measuring point in the aorta and the manometer is necessary.

*The efficiency of the technique used in the blood-pressure recordings.*

It is required of the apparatus employed that it should reproduce correctly the static pressures and pressure variations to be measured. The pressure curve recorded must be of such dimensions that the desired values can be measured with sufficient exactness. As a measure of the size of the pressure variations the variation of the capacity of the manometer is taken. The capacity increase for a given change in the distance between the two couples of condenser plates will be greatest when the primary distance is least. If the movable condenser plate is deformed under a variation of pressure, the change in distance will not be proportional to the actual pressure variations for a series of increasing pressures and the disproportion will increase with the degree of deformity. Therefore in the construction of the condenser manometer used care is taken to make the distance between the plates small and the rigidity of the movable plates great. In this way changes of volume and therefore of friction are diminished in the fluid-tube system necessary for the recording.

The fluid displacement in the system is determined by the volume alterations. These are measured for a series of static pressure values between 0 and 250 mm Hg. An alteration of



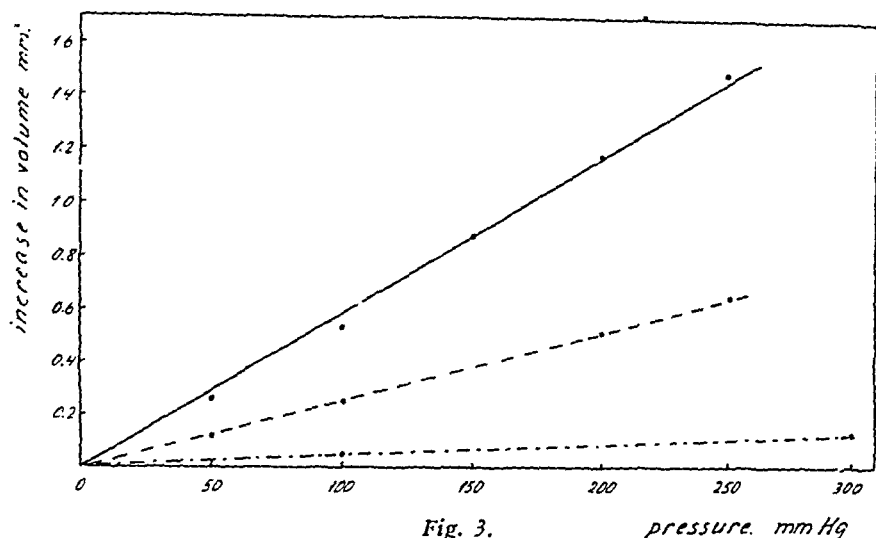


Fig. 3. pressure. mm Hg

Volume changes in the fluid-containing part of the recording system as a function of the pressure.

- glass tube with attached catheter filled with water.
- - - glass tube, condenser manometer and water.
- · - · glass tube with water.

abscissa: mm Hg.

ordinate: mm³.

100 mm Hg. causes a volume increase for the whole system of 0.75 mm³. The greater part, 0.5 mm³ is caused by the slight elasticity of the catheter (Fig. 3). The displacement of fluid in the distal part of the catheter will therefore amount to 0.3 mm for a pressure change of 100 mm Hg.

The complete pressure-recording system, including catheter, glass tube with citrate solution and condenser manometer, cable, high frequency circuit and oscillograph, is calibrated for static pressures by applying known pressures to the manometer, whereby it is shown that there is a linear relation between pressure values and deflections from 0 to 300 mm Hg. Gradual or sudden variations in temperature are without measurable influence upon the sensitivity of the pressure recording system.

For dynamic calibration the lower part of the catheter is inserted in a metal tube of which the other end is closed by the membrane of a condenser microphone of linear frequency characteristic. The closed system is filled with oil of the same

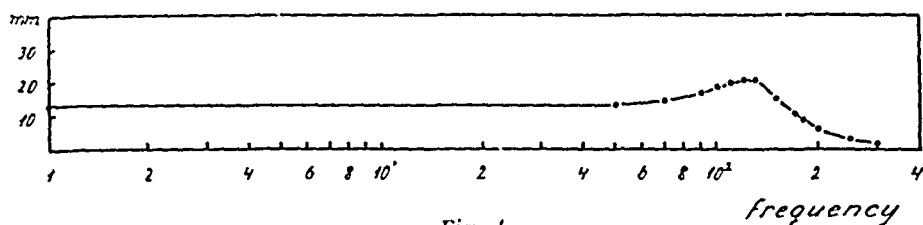


Fig. 4.

Dynamic calibration of the recording assembly.

abscissa: frequency in cycles per second, logarithmic scale.

ordinate: amplitude in mm.

viscosity as blood. The microphone membrane is made to vibrate at known frequencies by a laboratory oscillator and the periodically varying pressure amplitudes are recorded with an A.C. amplifier and oscillograph for different frequencies of oscillation. Figure 4 shows the frequency characteristic for a catheter length of 14 cm. When shorter catheters are used, the linear interval is unchanged but the damped part of the curve decreases in steepness and the frequencies recorded increase to 300 Hz., when the length of the catheter is 10 cm. As the frequencies of the blood-pressure variations investigated are less than 80 Hz., they always will be found in the linear registration area.

The condenser manometer proved to be without measurable hysteresis. This latter is a property of oscillographs of the type used, the torsion angle for a certain voltage being greater when the oscillograph has been exposed beforehand to a higher voltage. A detailed account of oscillographs of this type and their efficiency is given by *R. Elmquist (Porjé, thesis 1946)*. The oscillograph used in the present investigations was tested for hysteresis by recording the deflection for a series of increasing and thereafter decreasing pressures. Fig. 5 shows that the observed differences lie within the limits of experimental accuracy, as the distances on the recorded curve are measured to an accuracy of 0.5 mm, which corresponds to an accuracy of 1.5—2.0 mm in the determination of the pressure values. The greatest difference in pressure for a deflection of 6 cm is 10 mm Hg. The deflections mostly used are about 2 cm and the difference under such circumstances ca. 3 mm Hg.

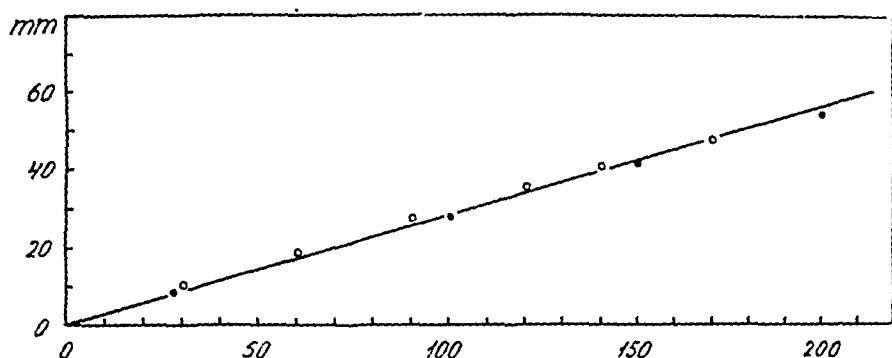


Fig. 5. *pressure mmHg.*

Static calibration. Deflection for a series of rising and thereafter falling pressures.

- rising pressures.
- ° falling pressures.

abscissa: mm Hg.

ordinate: mm deflection on the photographic paper.

The sensitivity used during the blood-pressure recordings has always been such that a 10 mm deflection on the photographic paper corresponds to a pressure value between 30 and 40 mm Hg. Variations in the capacity of the cable due to bending and pressure are eliminated completely for the part of the cable which moves during the experiments, partly by using a low-capacity cable and partly by inserting an autotransformer between the condenser manometer and the cable. To guard against the development of error in the apparatus after calibration the atmospheric pressure is recorded before and after every pressure reading during the experiments and, by means of a calibration capacity which gives for a known pressure a corresponding deflection, absolute values can be determined from the recorded pressure variations.

## CHAPTER IV

### THE VARIATIONS IN AORTIC PRESSURE DURING MUSCULAR WORK

#### *Transition from rest to work.*

After coupling to the recording assembly the animal is placed on the band of the tread-mill, where it stands still for 5 minutes before beginning exercise. The desired speed is attained in 4 to 5 seconds from the start, and the pressure variations are recorded continuously until the speed of running becomes constant, and usually in 10 to 20 seconds. 19 experiments have been performed on 8 different dogs. In 15 experiments the recording period includes the last 4—10 pulse-beats before the beginning of work; in the remaining experiments the resting pressure-pulse curves are recorded 1 minute before the starting of the tread-mill. From the pressure-pulse curves the systolic and diastolic blood-pressure values are measured for each pulse-beat and the pulse-rate is calculated from the duration of the single pulse-beat. The values so obtained are entered as of the time when the following pressure-pulse begins.

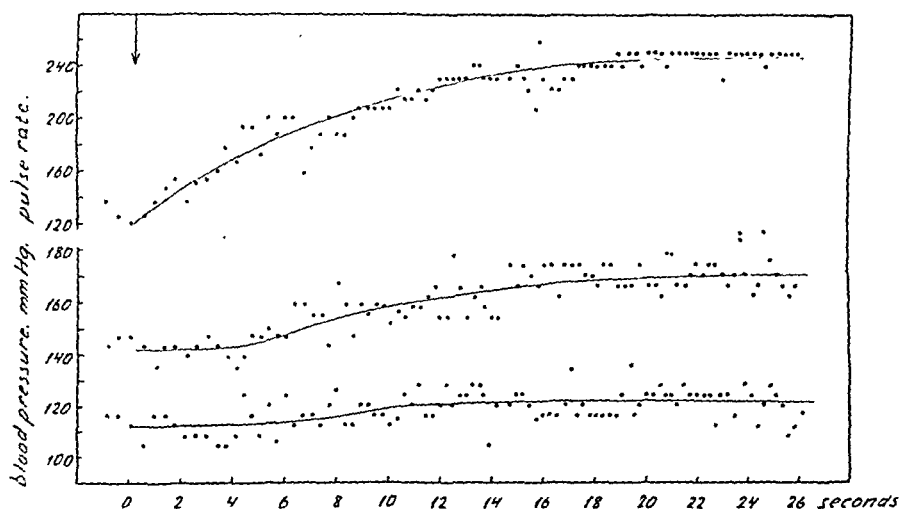
During rest the pulse-rate varies from one pulse-beat to another, the difference between the highest and lowest values being often 20 and sometimes 60 beats per minute. The blood-pressure values are more constant and differences of more than 10 mm Hg. are uncommon. In what follows the values observed during work are reckoned as raised or lowered when higher or lower than the extreme values observed during rest.

In table 2 the inclination of the running belt and the constant speed attained during the individual experiments are presented, and in addition the pulse-rate, systolic and diastolic blood-pressure and the pulse-pressure just before the beginning of work and at the second, fourth, . . . . 2.n seconds after exercise begins. The values quoted are the mean of the three pressure values nearest the point of time in question.

*Results.**Pulse-rate.*

The pulse-rate increases steeply at the beginning with isolated remissions which may be so marked that the resting value is regained. The succeeding rise is often slow in these cases. After the initial rapid increase in pulse-rate a steady decremental rise follows and after 10—20 seconds the pulse-rate attains a maximum (Figure 6). In the first few seconds the increase of pulse-rate is 20—40 per second. Relatively great differences are observed from one experiment to another, not only between different animals but also in the same animal in different experiments, with the same rate of work, so that it is impossible in this (transition) period to demonstrate any correlation between the rate of work on the one hand and the acceleration of pulse-rate and the maximum reached on the other.

After the beginning of work the rate in 4 experiments is slower for the first one or two pulse-beats than for the last

*Fig. 6.*

Pulse-rate, systolic and diastolic blood-pressures in the transition period from rest to work.

beginning of work

abscissa: seconds.

ordinate: mm Hg and heart beats per minute.

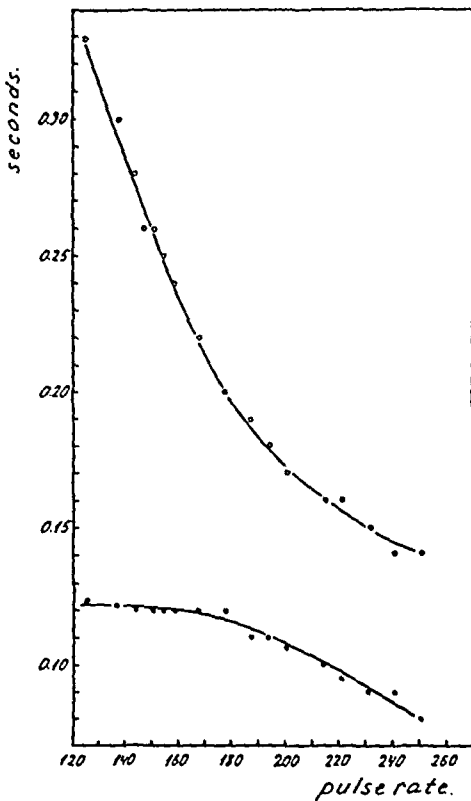


Fig. 7.

Duration of the ejection phase (below) and diastole after the closure of the valves (above) as a function of the pulse-rate.

abscissa: pulse-rate.

ordinate: duration in seconds.

during rest (30, 18, 10 and 8 beats per minute respectively). This fall in pulse-rate within the first second of beginning work is always preceded by a rise in pulse-rate in the last part of the resting period, from the moment when the command to start the treadmill is given, and is most probably attributable to the psychic influence of noise. The fall is followed immediately by a rise, so that the highest resting value for this experiment is exceeded within the first two seconds. In 9 of 15 experiments the first or second pulse-beat during work is shortened (Table 2, column 4). Only in one instance did the rise in pulse-rate occur after the fourth pulse interval. The time of occurrence of the first shortened pulse in the separate experiments is shown in

Table 2.

Dog No. and weight	I experiment No.	II speed of running	III inclination of the treadmill	IV The first shortened pressure pulse	V Time for rise in pulse-rate	VI Time for rise in systolic blood-pressure
I. 11,2 kg	1 .....	6 km/hour	5°			6,5 sec.
II. 12,1 kg	2 .....	6 km/hour	5°	2'	0,28 sec.	5,5 sec.
	3 .....	6 km/hour	5°			7 sec.
	4 .....	6 km/hour	5°			4 sec.
III. 7 kg	5 .....		13°	2'	0,32 sec.	2,5 sec.
IV. 8 kg	6 .....	6,5 km/hour	8°	4'	1,37 sec.	7 se
	7 .....	7,2 km/hour	8°	3'	0,44 sec.	5 sec.
	8 .....	6,7 km/hour	8°	4'	1,43 sec.	7 sec.

VII															
	before start	2' sec.	4' sec.	6' sec.	8' sec.	10' sec.	12' sec.	14' sec.	16' sec.	18' sec.	20' sec.	22' sec.	24' sec.	26' sec.	28' sec.
P.	156	166	180	180	182	177									
S.	126	165	170	171	174	174									
D.	87	130	137	133	135	133									
A.	39	35	33	38	39	41									
P.	214	224	230	233	237	247	240	243							
S.	148	149	149	145	142	149	149	148							
D.	108	109	111	105	111	109	111	115							
A.	40	40	38	40	31	40	38	33							
P.	200	205	224	222											
S.	156	167	167	168											
D.	116	127	126	130											
A.	40	40	40	38											
P.	230	219	224	230											
S.	154	152	155	158											
D.	118	118	124	124											
A.	36	34	31	34											
P.	196	204	214	214											
S.	120	125	139	137											
D.	90	99	104	102											
A.	30	26	35	35											
P.	143	152	161	183	234	243	250	257							
S.	133	131	132	133	136	142	155	157							
D.	97	95	93	87	94	92	104	108							
A.	36	36	39	46	42	50	51	49							
P.	131	154	179	192	191	207	230	230	219	240	247	250	250		
S.	141	141	137	151	156	155	162	154	171	170	170	171	166		
D.	109	109	112	114	117	114	122	116	115	116	121	124	121		
A.	32	32	25	37	39	41	40	38	56	54	49	47	45		
P.	127	149	174	191	204	207	211	204	227	219					
S.	137	142	143	149	150	163	163	175	175	178					
D.	108	113	113	109	119	120	111	117	125	128					
A.	29	29	30	40	31	43	52	58	50	50					



table 2 (continued)

Dog No. and weight	I	II	III	IV	V	VI
	experiment No.	speed of running	inclination of the treadmill	The first shortened pressure pulse	Time for rise in pulse-rate	Time for rise in systolic blood-pressure
	9 .....	7,2 km/hour .	12°	2'	0,43 sec.	4 sec
	10 .....	6,7 km/hour	14°	1'	0 sec.	7 sec
	11 .....	7,2 km/hour	16°	4'	1,26 sec.	3 sec
V. 11,2 kg	12 .....	6,5 km/hour	5°	10'	3,14 sec.	9 sec
VI. 22 kg	13 .....	7,3 km/hour	12°	2'	0,42 sec.	1 sec
	14 .....	13,7 km/hour	12°	2'	1,52 sec.	10 sec
VII. 16,3 kg	15 .....	3,5 km/hour	4°	1'	0 sek.	8 sec
	16 .....	9,3 km/hour	4°			3 sec.

VII

DETROIT START	2' sec.	4' sec.	6' sec.	8' sec.	10' sec.	12' sec.	14' sec.	16' sec.	18' sec.	20' sec.	22' sec.	24' sec.	26' sec.	28' sec.
148	185	200	196	214	180	224	233	225	237	250				
154	150	161	169	163	164	162	172	179	171	177				
114	111	119	123	112	116	120	125	128	121	126				
40	39	42	46	51	48	42	47	51	50	51				
162	182	164	169	177	200	230								
150	153	154	153	172	166	160								
119	118	109	116	120	118	112								
31	35	53	37	52	48	48								
138	158	191	158	167	187									
136	137	152	155	151	147									
99	102	118	116	114	114									
37	35	34	39	37	33									
171	177	193	204	209	212	224	224	227	230					
194	196	187	185	185	200	209	209	219	228					
160	159	153	155	149	166	173	167	164	166					
34	37	34	30	36	34	36	42	55	62					
138	191	198	205	216	161	136	151	174	174	198				
163	179	188	192	188	206	215	205	203	207	209				
136	142	154	158	154	162	162	166	157	159	156				
27	37	34	34	34	44	53	49	46	48	53				
93	134	157	169	200	212	209	203	198	230	250	253			
172	166	169	159	167	176	193	205	210	220	225	233			
130	128	120	119	134	138	145	146	159	160	173	182			
42	38	49	40	33	38	48	59	51	60	52	51			
80	130	150	174	180	177	142	132	135	147	134	138	158	150	167
113	118	111	110	117	125	139	134	133	132	133	134	137	131	130
63	67	61	59	65	75	73	71	70	74	80	81	90	85	83
50	51	50	41	52	50	66	63	63	58	53	53	47	46	47
106	178	148	174	197	154	172	191	187	174					
143	147	174	154	169	174	166	159	162	170					
83	111	107	100	120	114	111	103	109	109					
60	36	67	54	49	60	55	56	53	61					

table 2 (continued)

Dog No. and weight	I experiment No.	II speed of running	III inclination of the treadmill	IV The first shortened pressure pulse	V Time for rise in pulse-rate	VI Time for rise in systolic blood-pressure
VIII. 17,2 kg	17 .....	4,4 km/hour	7°	4'	0,94 sec.	2 sec.
	18 .....	6,9 km/hour	13°	1'	0,68 sec.	6,5 sec.
	19 .....	8,4 km/hour	13°	1'	0 sec.	8 sec.

Pulse-rate, systolic and diastolic blood-pressure and amplitude in the transition period from rest to work. The inclination of the treadmill, the constant speed obtained during the individual experiments are presented, and in addition the pulse-rate, systolic and diastolic blood-pressure and the ampli-

table 2, column 5. It occurs 0—3 seconds after the start (average 0.8 second). The increased pulse-rate produces mainly a shortening of diastole. As the end of systole is not indicated in the working pressure curve, the ejection phase is measured from the beginning of the steep systolic rise to the end of protodiastole, and the course of diastole is measured from then until the onset of the next ejection phase. Fig. 7 shows ejection phase and diastole as functions of the pulse-rate in a single experiment, where the pulse-rate rose from 120 to 250 in the course of about 20 seconds. Only when the pulse-rate in this particular experiment attains 180, is the ejection phase perceptibly shortened. The reason for quoting this time and not that of complete systole is that the beginning of the isometric contraction phase is not marked in the aortic pressure curve. The variations in pulse-rate for the experiment in question are shown in Fig. 6.

VII															
	<i>before start</i>	2.' sec.	4.' sec	6.' sec.	8.' sec	10.' sec.	12.' sec.	14.' sec.	16.' sec.	18.' sec.	20.' sec.	22.' sec.	24.' sec.	26.' sec.	28.' sec.
P.	185	209	205	219	211	240	250								
S.	180	184	190	197	190	184	198								
D.	136	140	140	141	126	117	127								
A.	44	44	50	56	64	67	71								
P.	169	185	200	240	260	231	253	237							
S.	181	180	181	187	195	198	203	197							
D.	129	128	128	128	132	129	122	105							
A.	52	52	53	59	63	69	81	92							
P.	134	212	237	250	260	250	247	240							
S.	182	181	181	194	202	211	208	202							
D.	127	121	116	129	138	137	125	130							
A.	55	60	66	65	64	74	83	72							

tude just before the beginning of work and at the 2nd — 4th — 6th — 8th — 10th — 12th — 14th — 16th — 18th — 20th — 22th — 24th — 26th — 28th second after beginning are given; besides the time for the first shortened pressure pulse and rise in systolic blood-pressure is quoted.

### *Systolic blood-pressure.*

The systolic blood-pressure rises and falls alternately 5—15 mm from pulse-beat to pulse-beat after the beginning of work, so that successive single values in some experiments lie respectively 1—2 mm above or below the values obtained at rest. When the pulse-rate in the individual experiment has risen substantially, real deviations from the resting values appear, as the pressure either immediately or after a transient fall begins to rise. The rise is followed in some cases by a constant pressure value, in others by a fall. In 2 experiments the pressure falls 4 and 5 seconds respectively after the beginning of work to a value 10 mm lower, whereupon it rises and passes the resting level 10 seconds after the start. In the other experiments the pressure value remains the same as under resting conditions until a rise occurs after 1—10 seconds (average 5 seconds). The

onset of the rise in the individual experiments is shown in table 2, column 6. In 5 cases the rise is maintained throughout the transition period. In the others one or more maxima and minima make their appearance on the steadily rising pressure curve until a constant pressure level is attained. This plateau in one case is succeeded by a gradual fall, but in the others is sustained during the remainder of the recording period. The rate of rise in pressure is not more than 10 mm per second and often less.

### *Diastolic blood-pressure.*

The diastolic blood-pressure follows the same course in the main as the systolic, but the gradient as a rule is less. In 4 experiments out of 19 a fall occurs 2 to 4 seconds after the beginning of work (Fig. 8), which reaches its lowest point in 6 to 7 seconds and amounts to 5, 8, 10 and 15 mm Hg. respectively. In the 2 cases where it was most distinct the pulse-rate had previously begun to rise steeply. In 3 cases the diastolic pressure rose 10 mm before the systolic pressure began rising. In the other experiments it rose synchronously with the systolic blood-pressure. In 2 cases a fall in the diastolic pressure occurred after 8 seconds of work.

### *The pressure amplitude.*

The rise in systolic blood-pressure is accompanied by a lesser rise in the diastolic, as described above, so that the amplitude in 14 of the 19 experiments increases up to 30 mm Hg. Great variations are seen, however, from one pulse-beat to another. In experiments No. 2 and 4 in table 2, the pressure amplitude diminished. The pulse-rate before these experiments was high. In the 14 experiments in which a definite amplitude increase occurred, abrupt increments sometimes took place. The cause of this in 12 cases was a rise in systolic blood-pressure, as a rule synchronously with the transition to constant or falling pulse-rate. In 5 cases the sudden increase in amplitude was due to a fall in the diastolic blood-pressure, which in 4 experiments was accompanied by a rise in pulse-rate. Similar increases in pulse-rate occurred without alterations in amplitude, however.

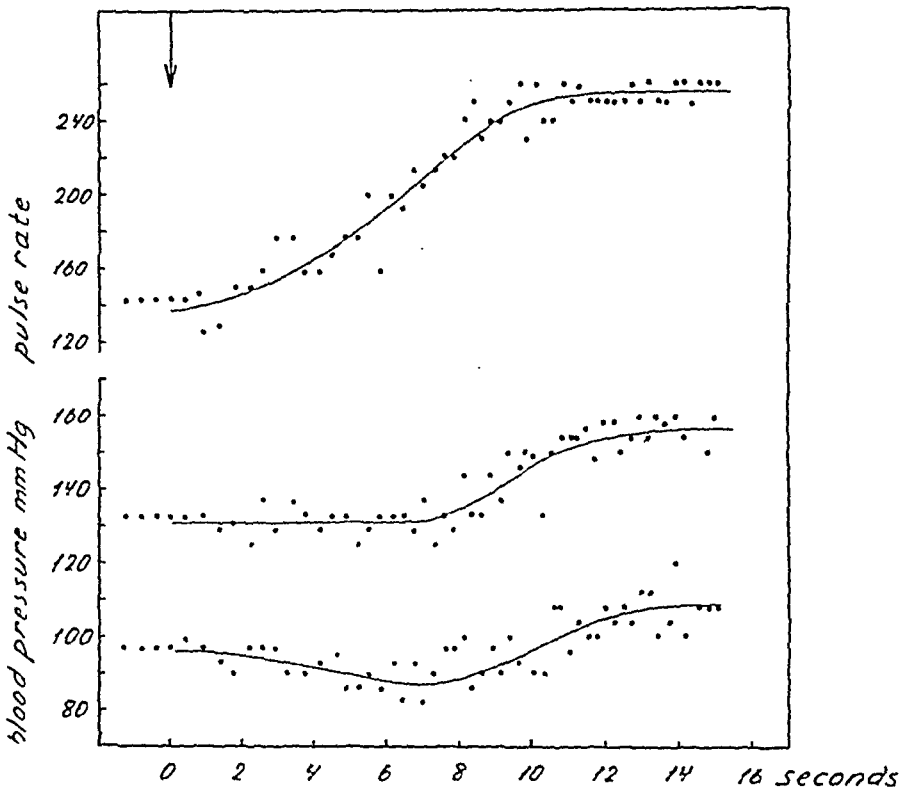


Fig. 8.

Pulse-rate, systolic and diastolic blood-pressures in the transition period from rest to work.

↓ beginning of work

abscissa: seconds.

ordinate: mm Hg and heart-beats per minute.

### *The mean pressure.*

As there exists the possibility that the mean pressure behaves differently from the systolic and diastolic blood-pressures because of alterations in the form of the pressure curve by muscular work, the mean pressure in 4 experiments is calculated from planimetric measurements of the single pressure-pulse in the first 3 or 4 seconds after work begins, since it is only in this period, before the limit values of the pressure pulse are increased, that deviations can be expected. In one case (No. 14 table 2) the values remained within the limits of the resting values and in 3 (Nos. 8, 15 and 17) a small rise was observed at the same time as or immediately after the very substantial

rise in pulse-rate. There was thus no indication of an initial fall in the mean pressure.

Summing up, the experiments show that the pulse-rate rises from the commencement of work before alterations in the blood-pressure can be demonstrated, and the course corresponds to the behaviour in man (*Neukirch* 1938) except that the rise does not occur instantaneously. The blood-pressure rises as a rule without a preceding fall, which is in accord with the results previously obtained in unanaesthetised dogs but conflicts with the blood-pressure course during electrically induced work on narcotised animals with damaged spinal cords.

### *The behaviour during work of longer duration.*

The influence of prolonged muscular work on blood-pressure and pulse-rate is investigated by recording the pressure variations in periods of 5—10 pulse-beats with short intervals during exercise of 5—45 minutes duration. As a rule several experiments are performed with each animal at differing constant rates of work, varied from experiment to experiment by changing either the inclination of the treadmill or the speed of running, to determine what influence these factors have upon the pressure variations.

The amount of work performed by running on the tread-mill at a known inclination and speed cannot be measured, as one component, the work of propulsion, is unknown. Besides it is chiefly of interest to investigate the dependence of pulse-rate and blood-pressure on the metabolic rate in the "steady state" under different working conditions. Therefore the oxygen intake has been determined for the type of work employed prior to the pressure measurements for all except one of the animals used. The speed of running for this one dog is controlled only 1 and 10 minutes after the beginning of work.

5 dogs have been used in the investigations and 18 experiments have been performed. The values for the systolic and diastolic blood-pressures and the pulse-rate for these dogs, after a resting period of 15—30 minutes, are given in table 3. The results obtained during work are given in tables 4—8.

Table 3.

Series No.	Systolic blood-pressure	Diastolic blood-pressure	Pulse-rate
1 .....	131 mm Hg.	96 mm Hg	133
2 .....	113 "	63 "	80
3 .....	139 "	84 "	140
4 .....	128 "	94 "	128
5 .....	136 "	101 "	113

Resting values for pulse-rate, systolic and diastolic blood-pressure recorded from the 5 dogs used in the working experiments in series 1-5. The recordings were made after the dogs had rested for 15-30 minutes.

### Results.

*Series 1.* Dog No. 4. Weight 8,2 kg.

6 experiments are performed over 3 days. Two experiments have been carried out each day, with an hour between the end of the first and the beginning of the second. The rates of work used and the values obtained for pulse-rate, blood-pressure and amplitude are given in table 4.

Table 4.  
*Series 1. Dog 8,2 kg.*

Experiment No	Inclination of the treadmill	Speed of running	Minutes after beginning of work	Blood-pressure in mm Hg.			Amplitude
				Pulse-rate	systolic	diastolic	
1.	8°	10,2 km/hour	1,5	242	154	107	47
			2	222	156	114	42
			3	221	165	120	45
			5,5	219	152	110	42
			7	217	168	131	37
			8,25	218	171	130	41
			11,5	187	170	125	45
			12,5	208	173	133	40
2.	8°	6,5 km/hour	0,5	249	149	102	47
			5,5	209	138	97	41
			8,0	218	138	101	37
			9,0	220	146	112	34
			11,0	232	138	97	41
			12,0	209	145	102	43



table 4 (continued)

Experiment No.	Inclination of the treadmill	Speed of running	Minutes after beginning of work	Blood-pressure in mm Hg.			Amplitude
				Pulse-rate	systolic	diastolic	
3.	8°	7.2 km/hour	1.5	192	155	107	48
			2.5	196	152	116	36
			4.5	193	168	126	42
			6.5	210	150	106	44
			8.5	186	144	100	44
			10.0	189	157	117	40
			12.0	175	152	108	44
			14.0	196	156	110	46
			16.0	187	150	111	39
			20.0	181	148	102	46
			22.0	185	165	122	43
			24.0	178	164	134	30
4.	8°	6.7 km/hour	2.5	183	151	112	39
			5.0	196	150	101	49
			5.75	197	152	110	42
			6.25	176	136	89	47
			10.0	200	146	102	44
			12.0	178	157	113	44
			15.0	178	160	115	45
			18.0	200	159	113	46
			20.0	197	149	108	41
			22.5	208	157	119	38
			24.0	193	157	114	43
			28.0	198	150	115	35
			31.0	207	153	112	41
			34.5	175	152	111	41
5.	12°	7.2 km/hour	1.0	246	173	113	60
			1.75	225	180	133	47
			2.0	229	176	134	42
			3.0	211	172	131	41
			6.0	217	170	122	48
			7.0	212	170	130	40
			9.0	213	171	129	42
			10.0	212	164	124	40
			11.0	200	167	123	44
			12.0	215	156	117	39
			14.0	215	159	118	41
			16.0	210	155	116	39

table 4 (continued)

Experiment No.	Inclination of the treadmill	Speed of running	Minutes after beginning of work	Pulse-rate	Blood-pressure in mm Hg.		Amplitude
					systolic	diastolic	
		7,2 km/hour	17,0	200	159	116	43
			20,0	208	167	119	48
			23,0	210	154	112	42
			25,0	204	160	122	38
			27,0	209	147	106	41
			28,0	206	150	106	44
			30,0	197	162	122	40
			32,0	193	153	117	36
			36,0	192	156	115	41
			40,0	197	149	112	37
			42,0	192	153	118	35
			45,0	190	154	111	43
6.	14°	6,7 km/hour	1,0	245	167	111	56
			1,5	203	170	121	49
			7,0	196	160	116	44
			8,5	195	158	114	44
			9,5	199	152	117	35
			11,0	203	153	114	39
			13,0	203	154	114	40
			15,0	183	146	101	45
			16,0	194	152	109	43
			19,0	192	146	107	39
			22,0	209	144	114	30
			24,0	196	147	107	40

Pulse-rate, systolic and diastolic blood-pressure and amplitude measured at different times in 6 working experiments on the same dog. The time interval from the beginning of work to the time for the individual measurement is given in minutes. The inclination of the treadmill and the speed of running are given for each experiment.

### *Series 2. Dog No. 7. Weight 16,3 kg.*

4 working experiments are performed on 3 days and the dog in all experiments ran quite freely. The oxygen intake per minute while running at different speeds and at 4°, 7°, and 13° inclination of the treadmill is seen from Fig. 9. The values obtained in the experiments are given in table 5.

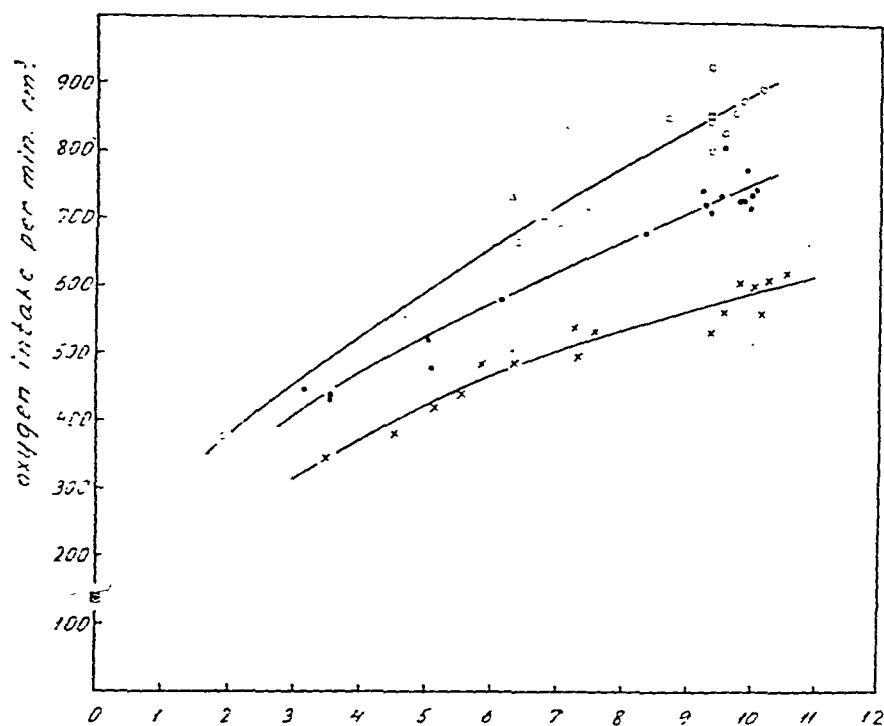


Fig. 9. *speed. km/hour*

Oxygen intake as a function of the speed of running at 4° (X), 7° (·) and 13° (°) inclination. Series 2.

abscissa: speed in km per hour.

ordinate: oxygen intake in cm³ per minute.

Table 5.  
Series 2. Dog 16,3 kg.

Experiment No.	Inclination of the treadmill	Speed of running	Minutes after beginning of work	Pulse-rate	Blood-pressure in mm Hg.			Amplitude
					systolic	diastolic		
1.	4°	3,25 km/hour	0,5	167	130	83		47
		3,25	2,0	110	150	94		56
		3,25	4,5	130	166	105		61
		3,30	5,5	108	140	77		63
		3,30	7,0	126	126	70		56
		3,30	9,5	94	137	76		61
		3,25	14,0	125	139	71		68
		—	16,0	123	138	77		61

Table 6.  
Series 3. Dog 17 kg.

Experiment No.	Inclination of the treadmill	Speed of running	Minutes after beginning of work	Pulse-rate	Blood-pressure in mm Hg.		Amplitude
					systolic	diastolic	
1.	7°	4,4 km/hour	2,5	248	215	149	66
		4,6 "	4,5	252	227	161	66
		4,9 "	5,5	259	226	161	65
		5,4 "	8,25	238	240	167	73
		5,4 "	9,5	260	233	158	75
		5,7 "	11,3	243	242	175	67
		5,7 "	12,0	273	227	160	67
		5,7 "	12,40	246	224	156	68
		5,7 "	15,0	265	229	143	76
		5,6 "	15,45	257	234	159	75
2.	13°	6,9 km/hour	1,5	237	193	130	63
		6,9 "	2,0	256	210	155	55
		5,0 "	3,0	231	183	137	46
		5,0 "	4,0	220	186	141	45
		5,0 "	4,5	204	184	127	57
		6,1 "	5,75	212	214	154	60
		6,5 "	7,0	222	204	143	61
		6,5 "	9,5	225	204	147	57
		6,5 "	11,0	234	204	144	60
3.	13°	8,6 km/hour	0,25	240	202	130	72
		8,6 "	1,0	230	207	145	62
		8,6 "	5,0	240	227	178	49
		8,6 "	6,5	254	224	156	68
		9,0 "	7,0	250	224	164	60
		9,0 "	12,0	250	229	181	48

Pulse-rate, systolic and diastolic blood-pressure and amplitude measured at different times in 3 working experiments on the same dog. In the first experiment the dog had diarrhoea and was very much pre-occupied with the surroundings. The time interval from the beginning of work to the time for the individual measurement is given in minutes. The inclination of the treadmill and the speed of running are given for each experiment.

Series 4. Dog No. 9. Weight 13,7 kg.

Three working experiments are performed. Fig. 11 shows the oxygen intake for work of different rates at 7° and 13° inclination. The experimental results are shown in table 7.

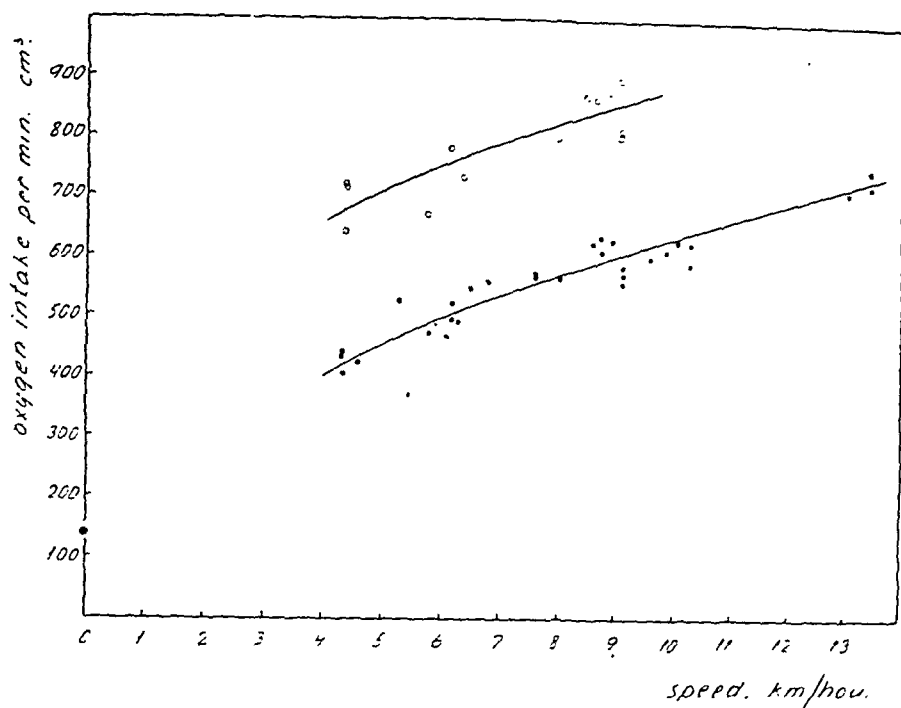


Fig. 10.

Oxygen intake as a function of the speed of running at 7° (•) and 13° (°) inclination. Series 3.

abscissa: speed in km per hour.

ordinate: oxygen intake in cm³ per minute.

### Series 3. Dog No. 8. Weight 17 kg.

3 working experiments were performed. During the first the dog had diarrhoea and was very much pre-occupied with its surroundings. During the later experiments it was more tranquil, but under the influence of the manipulations necessary for the recording of the atmospheric pressure it invariably slowed down the speed of running when the cocks in the glass-tube below its neck were turned. On this account the pressure variations were all recorded before the determination of the atmospheric pressure. Fig. 10 shows the oxygen intake during running at 7° and 13° inclination. The values obtained for blood-pressure and pulse-rate in the experiments are shown in table 6.



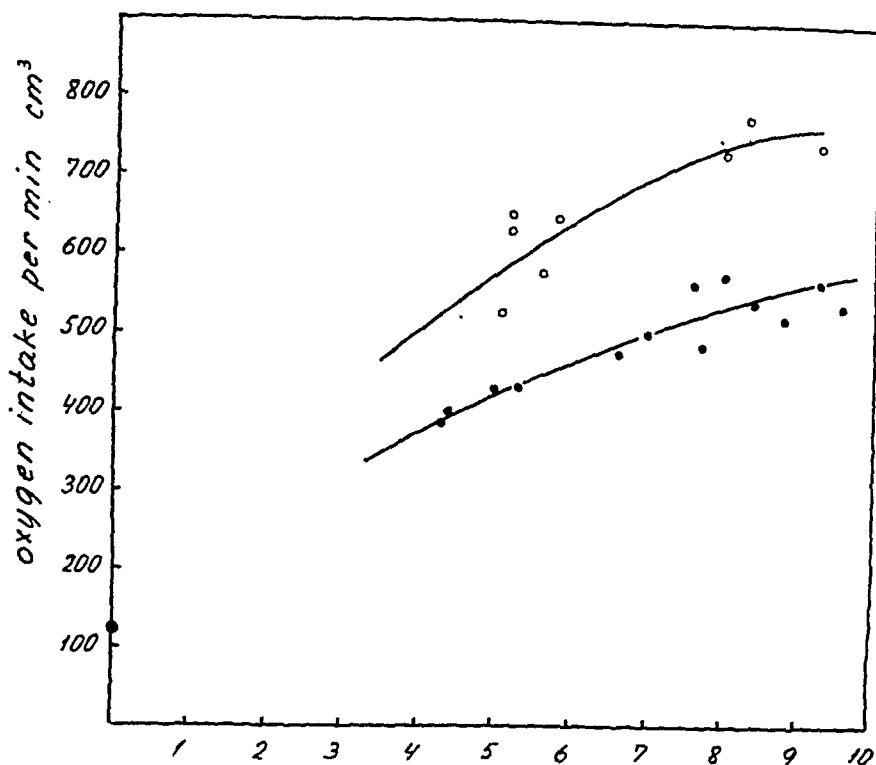


Fig. 11. *speed km/hour.*

Oxygen intake as a function of the speed of running at 7° (•) and 13° (°) inclination. Series 4.

abscissa: speed in km per hour.

ordinate: oxygen intake in cm³ per minute.

*Series 5. Dog No. 10. Weight 14 kg.*

Two working experiments are performed. The oxygen intake for work at 7° and 10° inclination is shown in Fig. 12. The results are given in table 8.

In 10 experiments the pressure variations are measured both in the transition period from rest to work and in the first minutes of constant muscular work, and from these measurements it can be seen that the maximal pulse-rate attained just after the beginning of work is succeeded by a fall to a value 30 to 60 beats lower (Fig. 13). The fall begins 20—30 seconds after the beginning of work and ceases within 2—3 minutes. Only in one experiment did the pulse-rate remain at the initial

Table 7.  
Series 4. Dog 13,7 kg.

Experiment No.	Inclination of the treadmill	Speed of running	Minutes after beginning of work	Pulse-rate	Blood-pressure in mm Hg.		Amplitude
					systolic	diastolic	
1.	7°	4,22 km/hour .....	8,0	165	151	107	44
2.	13°	4,22 km/hour .....	0,15	221	171	122	49
		4,22 „ .....	0,30	230	169	125	44
		4,22 „ .....	1,0	226	177	136	41
		4,4 „ .....	4,0	230	171	121	50
		4,4 „ .....	5,0	240	170	127	43
		4,9 „ .....	6,0	250	162	119	43
		4,9 „ .....	8,0	240	164	126	38
		4,9 „ .....	10,0	235	166	129	37
3.	13°	5,4 km/hour .....	3,0	222	175	133	42
		5,7 „ .....	5,0	235	172	128	44

Pulse-rate, systolic and diastolic blood-pressure and amplitude measured at different times in 3 working experiments on the same dog. The time interval from the beginning of work to the time for the individual measurement is given in minutes. The inclination of the treadmill and the speed of running are given for each experiment.

level throughout the working period. The dog in this experiment had diarrhoea and ran very irregularly. The systolic blood-pressure, which likewise rises in the transition period, in 5 cases reaches a maximum and then falls by as much as 30 mm Hg to a level within the first three minutes. In the other experiments the initial rise continues, but with diminishing steepness, until after 3—7 minutes it reaches a level 10—60 mm Hg. above the value reached in the first half-minute. The diastolic blood-pressure follows with insignificant deviations the course of the systolic pressure, so that the amplitude does not change. In one experiment where the work was very light, the diastolic blood-pressure falls below the initial level. As the cassette used for collecting the exposed film is too small to allow of the pressure variations being recorded so frequently that the detailed course of the pulse-rate and blood-pressure during the first minutes of



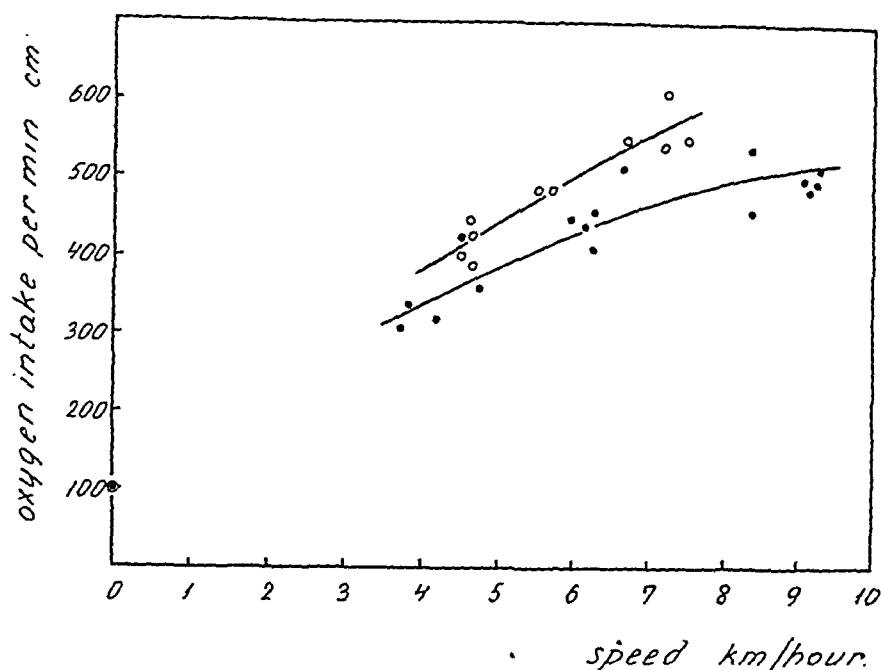


Fig. 12

Oxygen intake as a function of the speed of running at 7° (•) and 10° (°) inclination. Series 5.

abscissa: speed in km per hour.

ordinate: oxygen intake in cm<sup>3</sup> per minute.

work can be examined, it is impossible to determine the time of onset of the observed sudden variations sufficiently exactly to decide if they are dependent upon the rate of work. The level reached by the pulse-rate and blood-pressure in the first minutes of work is in most experiments preserved during the remainder of the work irrespective of its duration, provided the speed of running is unchanged. The pulse-rate often shows deviations of 5 per cent (rarely 10 per cent) of the mean value, corresponding to a maximum of 20 beats, while the variations in the systolic and diastolic pressures are smaller, as a rule not more than 5 mm Hg. Deviations of as much as 15 mm Hg. can, however, be seen, especially in the diastolic pressure course, as an increase in pulse-rate is often accompanied by a diastolic pressure fall. In 2 of the longest experiments (Series 1, Nos. 5 and 6) the blood-pressure falls 15 mm during work lasting 25 and 45 minutes respectively. The cause of this is obscure, but

Table 8.  
Series 5. Dog 14 kg.

Experiment No.	Inclination of the treadmill	Speed of running	Minutes after beginning of work	Pulse-rate	Blood-pressure in mm Hg.		
					systolic	diastolic	Amplitude
1.	7°	4,4 km/hour .....	1,5	210	153	114	39
		4,4 » .....	4,0	220	162	122	40
		4,4 » .....	6,0	193	162	124	38
		4,4 » .....	11,0	240	160	128	32
		4,4 » .....	17,0	200	164	127	37
2.	10°	5,2 km/hour .....	0,3	220	171	133	38
		5,2 » .....	1,0	230	167	120	47
		5,2 » .....	1,5	214	168	124	44
		5,2 » .....	5,0	200	179	136	43
		5,2 » .....	8,0	250	185	142	43
		5,2 » .....	9,0	260	186	142	44
		5,2 » .....	11,0	250	184	147	37

Pulse-rate, systolic and diastolic blood-pressure and amplitude measured at different times in 2 working experiments on the same dog. The time interval from the beginning of work to the time for the individual measurement is given in minutes. The inclination of the treadmill and the speed of running are given for each experiment.

possibly the animal has gradually reduced the speed of running, which in these experiments was controlled only at the 1st and 10th minutes.

The recorded values for pulse-rate and blood-pressure during work in the "steady state" rise with the rate of work, and for the moderate outputs of work here investigated are often located at 200—240 in the case of the pulse-rate, while the systolic blood-pressure reaches 180—240 mm Hg. The bearing of the rate of work upon the level of pulse-rate and blood-pressure reached in the experiments is illustrated by Figs. 14 and 15, which show the relationship between pulse-rate, systolic and diastolic blood-pressure, and oxygen intake during work in the "steady state" of varying intensity. In addition the lowest values for pulse-rate and blood-pressure during rest are given as a function of the resting (but not basal) metabolism.

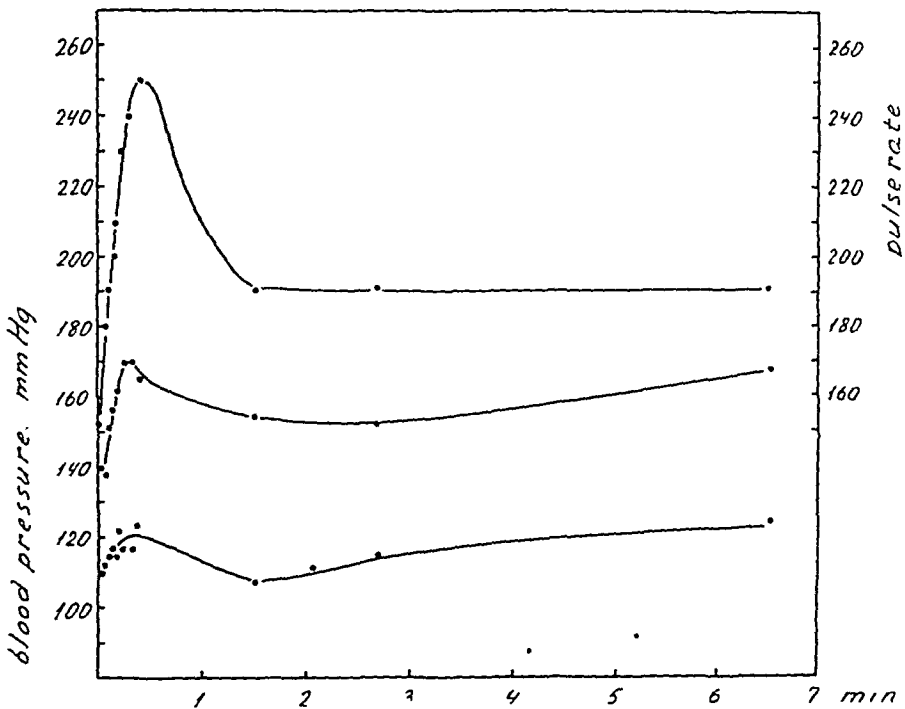


Fig.13.

Pulse-rate, systolic and diastolic blood-pressures in the first minutes of work.  
abscissa: minutes.

ordinate: beats per minute and mm Hg.

From the figures can be seen that: —

- 1) The rise of pulse-rate and blood-pressure is dependent upon the work performed.
- 2) It is of no consequence whether this is pre-eminently lifting or propulsion work.
- 3) *There exists a linear correlation between systolic and diastolic blood-pressures and oxygen intake during work and a somewhat less constant correlation between oxygen intake and pulse-rate.*
- 4) The rise in systolic and diastolic pressure is the same, so that the amplitude is unchanged.

The other series of experiments give corresponding results. The rates of work employed are different in the 5 series, and the oxygen intake per kg body weight varies between 20 and

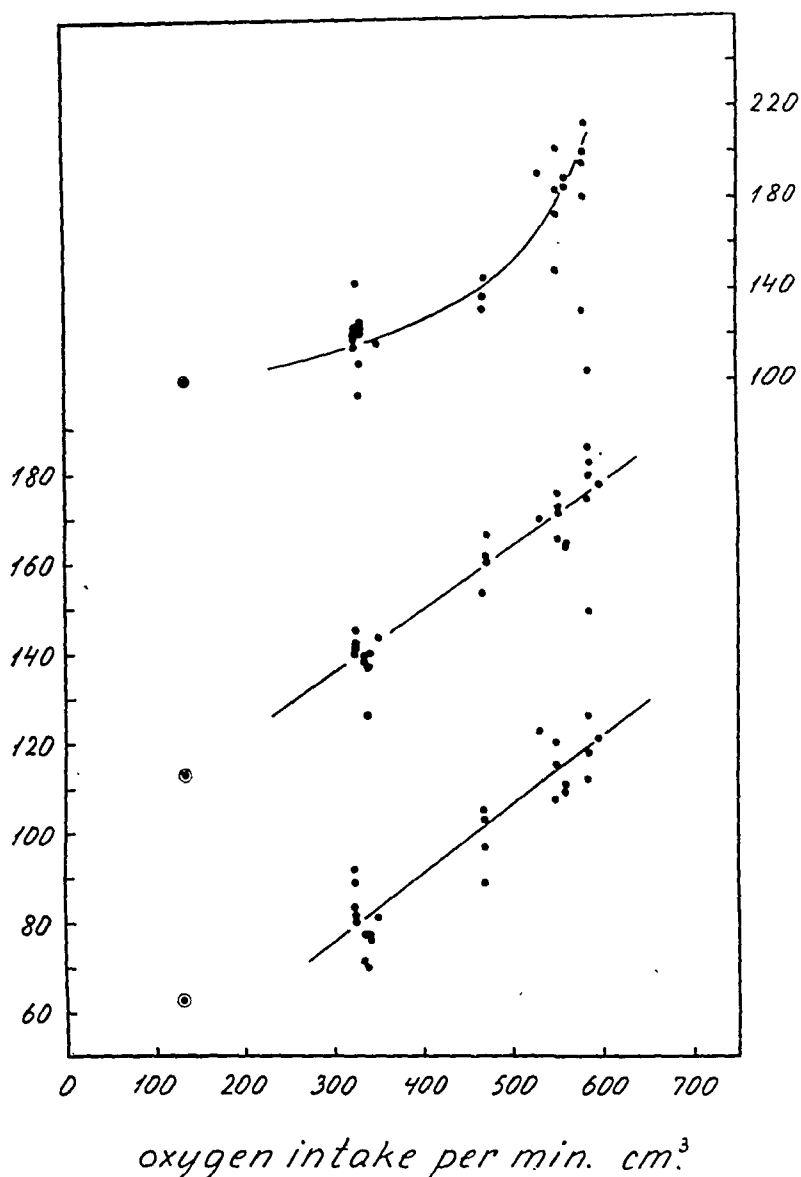


Fig. 14.

Pulse-rate, systolic and diastolic blood-pressures as functions of the oxygen intake during work in the "steady state" in series 2. The lowest resting values found in this particular dog are given to the left of the figure.

abscissa: oxygen intake in  $\text{cm}^3$  per minute.

ordinate: beats per minute and mm Hg.

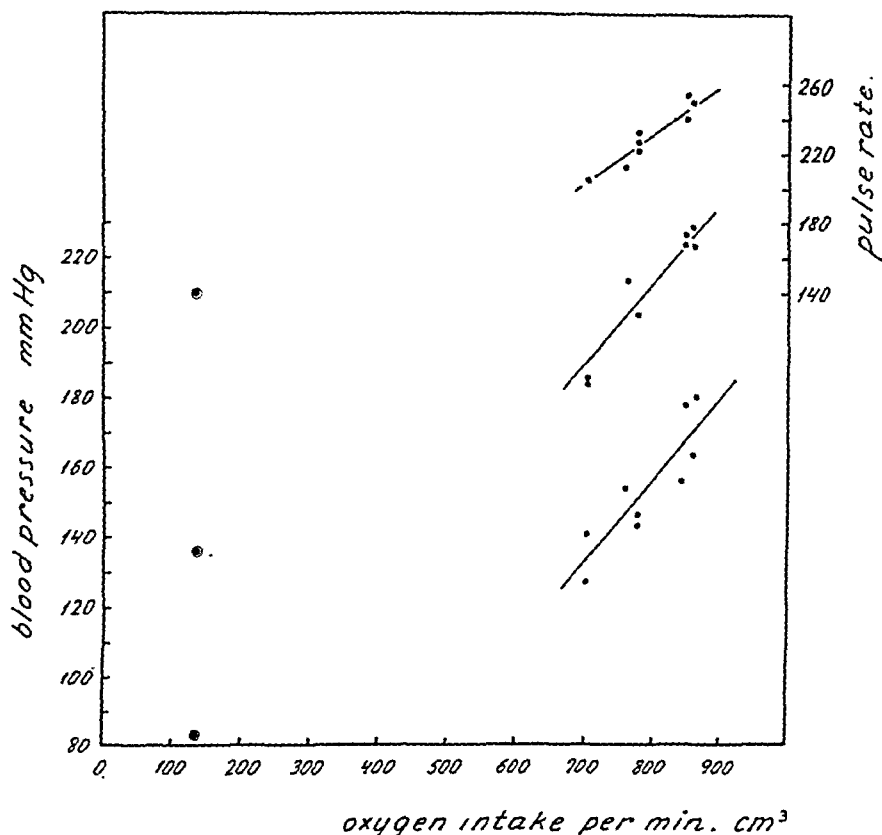


Fig. 15.

Pulse-rate, systolic and diastolic blood-pressures as functions of the oxygen intake during work in the "steady state" in series 3. (Experiment No. 2 and 3). The lowest resting values found in this dog are given to the left on the figure. abscissa: oxygen intake in  $\text{cm}^3$  per minute. ordinate: beats per minute and mm Hg.

$55 \text{ cm}^3$  per minute, which corresponds to an increase upon the resting metabolism of 250—700 per cent. The absolute pressure change for an increase in oxygen intake of  $1 \text{ cm}^3$  per minute varies in the different series of investigations between 1,5 and 3,4 mm Hg. The relative pressure rise as a function of oxygen intake is investigated for all experiments together in this way — the absolute systolic and diastolic blood-pressures for each series are either measured or calculated by interpolation for an oxygen intake of  $34 \text{ cm}^3$  per minute, which lies at or only a little below the working range used in all 5 series of experiments. From

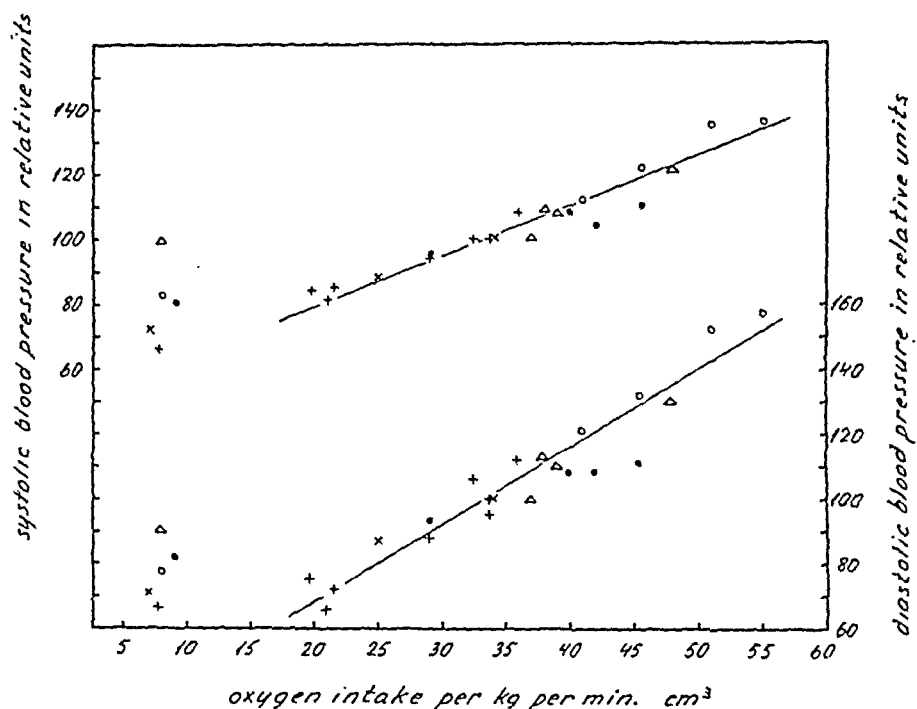


Fig. 16.

Relative systolic and diastolic pressures from all series as functions of the oxygen intake per kg per minute. The values given for the individual animal are the mean of all values for the oxygen intake in question.

△ Series 1. + Series 2. ○ Series 3. • Series 4. × Series 5.

abscissa: oxygen intake per kg per minute in  $\text{cm}^3$ .

ordinate: pressure values as per cent of values corresponding to an oxygen intake of  $34 \text{ cm}^3$  per kg per minute.

these values the percentage variations in systolic and diastolic blood-pressures are calculated for the individual animal and plotted in a common co-ordinate system as a function of the oxygen intake (Fig. 16). The figure shows that the relation between relative systolic and diastolic pressure values from all the experiments and the oxygen intake is practically linear in the working range investigated. That the gradient is greatest in the case of the diastolic rise is due to the diastolic blood-pressure being lower than the systolic, so that a definite deviation from the initial value, when expressed as a percentage, is greater for diastolic values than for systolic. The relative values for the resting pressure are plotted on Fig. 16. They are most often higher than expected, if it be assumed that the pres-

sure increase from the resting level is linear. This may be due to the determinations during rest not being carried out under basal conditions or to the operation of other factors than the metabolic rate. A comparison is made between the working values observed at  $8^{\circ}$  inclination for dog No. 4 and the oxygen intake at the same speed of running in dog No. 7 at  $7^{\circ}$  inclination. The two animals have about the same build, which makes the comparison permissible. The oxygen intake per kg body weight during work at a given rate is greater in short-legged dogs than in long-legged dogs. Variations in the two groups are only small.

Experiment No. 1 in series 3 shows that the condition of the dogs greatly influences the pressure level and that to obtain reliable results a good general condition and indifference to the surroundings are required. The work performed has always been moderate, the oxygen intake in no case exceeding that of the resting dog by more than 700 per cent. Hence it is impossible from the present experiments to form any conclusions about how the pulse-rate and blood-pressure respond to heavy work.

The observed course of the pulse-rate from the maximum reached in the transition period to the "steady state" value, which is maintained so long as the rate of work remains unchanged, corresponds to the behaviour observed in investigations on man and may be due to over-compensation for the demands made upon the circulation with a subsequent adjustment to the requisite values. The rise in the aortic systolic pressure agrees well with the values observed in man for the indirectly measured systolic blood-pressure during corresponding rates of work. The directly determined aortic diastolic pressure lies much above the diastolic pressure measured by auscultation under the same conditions (Table 9). Both direct measurements and indirect determinations show that the systolic blood-pressure always rises to a "steady state" value, the height of which depends upon the rate of work. The fairly uniform rise found by the two methods suggests that the indirectly measured systolic pressure during work corresponds roughly to the real scale, but no opinion can yet be given of the significance of the fact that the oscillometrically determined flow-pressure rises only a little or remains

Table 9.

Oxygen intake cm <sup>3</sup> /min	Rate of work mkg/min	Blood-pressure in mm Hg.			
		systolic auscultat	diastolic auscultat	max. occlusion pressure	max. flow- pressure
300	Rest	114	75	117	77
1300	410	149	72	154	96
2140	820	172	68	178	100
2560	1030	191	65	185	100
3000	1235	186	55	184	92
3440	1440	200	62	205	100

Blood-pressure values obtained in man during work by means of indirect methods. (Em. Hansen 1937).

unchanged under such circumstances. The determination are not an expression for the mean pressure as the directly measured pressure-level is elevated under corresponding working rates. The height of the diastolic pressure during work as determined by auscultation can be considered entirely valueless.

#### *The transition from work to rest.*

The aortic pressure variations were recorded continuously in 10 experiments on 6 dogs, running at full working speed and for 4—20 seconds after stopping the motor, which drives the belt of the treadmill. The work does not stop instantaneously but in the course of a few seconds, depending on the braking of the belt by the dog.

In table 10 the readings for the inclination of the treadmill and the speed and duration of running in the preceding working period in each experiment are given, together with the pulse-rate, systolic, diastolic and pulse pressures at the second, fourth. . . . . 2.n seconds after running ceases, calculated as the mean value of the 3 pulse beats nearest the time in question.

#### *Pulse-rate.*

The pulse-rate as a rule falls steeply at first, later more slowly with wide deviations from the mean value, and at the end of the recording period lies considerably above the resting level. In one experiment the pulse-rate rose in the very short recording period from 220 to 240. In the other 9 experiments.





6.	7,2 km/hour	12°	46	Min.	P. 148	200	214	214	207	200
					S. 154	173	167	158	159	158
					D. 114	112	118	109	104	104
					A. 40	61	49	49	55	54

V. 11,2 kg	7.	6 km/hour	5°	5	Min.	P. 154	273	245	220	214	209	233	227	230	209
						S. 192	216	220	217	210	199	205	206	201	193
						D. 156	180	182	177	178	167	178	174	177	166
						A. 36	36	38	40	32	32	27	32	24	27

VII. 16,3 kg	8.	3,25 km/hour	4°	30	Min.	P. 80	144	150	107	127	138	112	93	89	103	97	112
						S. 113	141	139	143	133	131	139	140	139	140	197	135
						D. 63	92	96	90	87	84	89	86	87	93	87	89
						A. 50	49	43	53	46	47	50	56	52	47	50	46

9.	9,3 km/hour	4°	20	Min.	P. 106	181	200	132	132	196	193	177	187
					S. 143	202	206	200	204	209	210	218	202
					D. 83	149	167	141	155	164	172	175	152
					A. 60	53	39	59	49	45	38	43	50

VIII. 17,2 kg	10.	6,5 km/hour	13°	11	Min.	P. 169	234	234	240
						S. 181	204	195	191
						D. 129	144	144	135
						A. 52	60	51	56

Pulse-rate, blood-pressure and amplitude 2, 4, — — seconds after stopping the mill. Besides the inclination of the tread mill and speed of running before the case of work the duration of work is given.

where a fall occurred, a value 20 to 70 beats below the working value was reached in 4—6 seconds. Afterwards there appear deviations, at first large but steadily diminishing, of the pulse-rate from the mean value, which remains constant or falls a little. The readings after 10 seconds lie 25—90 beats above the resting level, and in 5 cases where the recording continued for 15 seconds, the resting level was reached in only one, while the rate in the others still remained 10—50 beats higher than this. No correlation exists between the working level and the first steep fall in rate, which is independent of the resting level also. The fall in the whole recording period is independent of the duration of work, but the experiments on dogs Nos. IV and VII, where the behaviour is investigated after work of different rates, show that the total fall in pulse-rate is least after the highest rates of work.

### *The systolic blood-pressure.*

The systolic blood-pressure begins to fall within 4 seconds of stopping the treadmill. The resting level or a level little higher than this is regained in 3—14 seconds, and as a rule is maintained during the remainder of the recording period. In 6 experiments the fall in blood-pressure occurred in the first 2 seconds and in the other 4 about the 3rd or 4th second. The fall amounts to 15—30 mm Hg. in all (2—5 mm per second) and is most marked in the experiments where it lasts longest. In 2 experiments on dog No. VII it was followed by a rise of 30—40 mm Hg. above the resting level, while in the other experiments the fall ended in a plateau, the same as the resting level in 5 experiments and 5—10 mm above this in 3 experiments.

No correlation between pressure fall per second and the rate or duration of the preceding work could be demonstrated. In 6 experiments the changes in blood-pressure occurred subsequently to the fall in pulse-rate, and in 2 experiments pulse-rate and blood-pressure fell simultaneously. In the 2 other experiments the pulse-rate rose while the blood-pressure fell steeply.

*Diastolic blood-pressure and pulse-pressure.*

The diastolic blood-pressure follows in the main the systolic. However, it is observed that it decreases without a corresponding change in the systolic blood-pressure when the pulse-rate rises, so that the pulse-pressure increases. Most commonly the pulse-pressure decreases upon the cessation of work simultaneously with a decrease in pulse-rate.

From the investigations described it appears that the pulse-rate in the transition from work to rest has a more variable course than the blood-pressure. It decreases as a rule before the blood-pressure, and rises in some cases with a steep pressure fall, while marked deviations occur on the falling pulse curve without corresponding alterations in blood pressure. In 7 out of 10 experiments the systolic and diastolic blood-pressures fall to the resting level, or values not far from this, within 5—10 seconds. The fall occurs therefore essentially faster than the rise at the beginning of work, which is in agreement with the results obtained by indirect measurements of the blood-pressure in man (*Bowen* 1904). In only one experiment did the pulse-rate regain its initial level and rise again. In the other experiments it remained essentially above the resting level throughout the recording period.

## CHAPTER V

### THE SHAPE OF THE AORTIC PRESSURE CURVE AT REST

The aortic pressure course has been recorded in 13 dogs after a period of 15—30 minutes rest. The systolic pressure varied between 113 and 193 mm Hg. (147 mm average), the diastolic blood-pressure was 87—156 mm Hg. (102 mm average), and the pulse rate 77—156 (120 average) (Table 11). Fig. 17 shows a

*Table 11.*

Pulse-rate	Systolic blood-pressure	Diastolic blood-pressure	Amplitude
156 .....	126 mm Hg.	87 mm Hg.	39 mm Hg
77 .....	141 "	89 "	52 "
120 .....	153 "	102 "	51 "
133 .....	141 "	105 "	36 "
111 .....	193 "	148 "	45 "
133 .....	131 "	96 "	35 "
154 .....	192 "	156 "	36 "
103 .....	166 "	130 "	36 "
80 .....	113 "	63 "	50 "
140 .....	139 "	84 "	55 "
123 .....	145 "	75 "	70 "
128 .....	128 "	94 "	34 "
113 .....	136 "	101 "	35 "

Resting values for pulse-rate, systolic and diastolic blood-pressure and amplitude recorded from 13 different dogs. The single values are mean values obtained by measuring 5—10 continuous pressure-pulses.

characteristic pressure curve. The following notation will be used in the description. From the start of the ejection phase (A) the pressure rises very quickly to begin with and afterwards (from B) more slowly, to a maximum, C. Hereafter the pressure decreases until the end of systole (D). In the succeeding proto-diastole (DE) the pressure falls steeply again until the moment

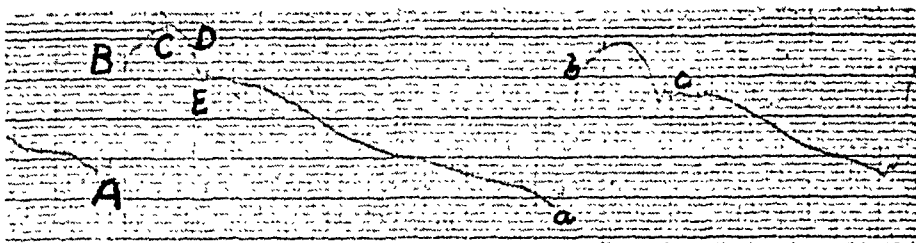


Fig. 17.

Aortic pressure curves in resting unanaesthetised dog.

- A: beginning of ejection phase.
- B: transition from steep to slow systolic rise.
- C: systolic maximum pressure.
- D: end of systole.
- E: end of proto-diastole.
- a: pre-oscillations.
- b: initial oscillations.
- c: post-oscillations.

Time mark  $1/50$  &  $1/10$  second.

the semilunar valves close (E). Except in one experiment, where the resting pressure was very high, a positive pressure wave occurs in the next part of the diastolic pressure curve, so that the pressure at first rises insignificantly above the value found at E before falling evenly during the remainder of the period. The duration of the separate phases and the pressure variations in them are measured from 20 pulse beats recorded in 10 different dogs (Table 12). The systolic blood-pressure varied in this material between 110 and 192 mm Hg. and the pulse-rate calculated from the single pulse beat was between 90 and 170. The pressure rise AB is 25–65 mm Hg., at the rate of 0.8–1.5 mm/ms. The rise is 50–93 per cent (average 81 per cent) of the total pressure rise and lasts 20–40 ms. In the period BC the pressure rises 3–22 mm in 30–70 ms (0.06–0.35 mm/ms). In CD the pressure falls 3–10 mm in 20–60 ms (0.08–0.5 mm/ms). The pressure fall in the period DE amounts to 6–20 mm in 10–30 ms (0.4–1.2 mm/ms). The pressure fall before the closure of the aortic valves as a rule is less than half of the total pressure amplitude in each cycle. Only in one experiment is the proportion greater (0.58). The pressure fall in diastole from E to A varies between 0.04–0.13 mm/ms and is remarkably

Date	Pressure alteration in mm Hg.	Duration in seconds	Pressure alteration in mm Hg.	Duration in seconds	Pressure alteration in mm Hg.	Duration in seconds	Pressure alteration in mm Hg.	Duration in seconds	Blood-pressure	Pulse-rate		
26/6 45	28	0.04	4	0.04	7	0.03	6	0.015	30	0.24	141/105	162
1. ....	30	0.04	5	0.06	4	0.02	6	0.015	25	0.26	135/100	158
27/7 45	26	0.02	18	0.07	3	0.04	9	0.02	36	0.37	189/145	120
2. ....	22	0.02	22	0.06	6	0.04	12	0.02	20	0.32	189/151	130
28/11 45	30	0.02	6	0.05	6	0.04	12	0.02	18	0.30	133/97	143
3. ....	30	0.02	6	0.05	6	0.04	12	0.02	18	0.30	133/97	143
13/12 45	27	0.02	9	0.06	6	0.04	12	0.01	34	0.26	192/156	154
4. ....	27	0.02	9	0.06	6	0.04	12	0.01	34	0.23	192/156	167
30/1 46	36	0.06	3	0.05	3	0.04	12	0.02	24	0.41	166/177	91
5. ....	36	0.05	3	0.05	6	0.05	12	0.02	13	0.34	166/135	115
24/9 46	39	0.035	9	0.065	5	0.05	8	0.02	44	0.63	116/59	75
6. ....	44	0.04	6	0.06	6	0.05	9	0.02	25	0.48	110/70	94
4/11 46	48	0.04	4	0.04	30	0.05			26	0.31	140/84	130
7. ....	50	0.04	6	0.03	32	0.06			24	0.33	140/84	130
29/3 47	65	0.04	7	0.06	10	0.02	15	0.02	40	0.35	145/80	120
8. ....	50	0.04	15	0.05	5	0.04	20	0.03	50	0.40	142/67	109
10/6 47	20	0.02	14	0.04	5	0.03	9	0.02	20	0.30	128/94	143
9. ....	30	0.03	6	0.03	6	0.06	7	0.01	23	0.40	130/94	115
26/6 47	28	0.03	4	0.04	4	0.04	8	0.01	32	0.54	132/88	91
10. ....	36	0.03	4	0.04	8	0.04	12	0.015	20	0.31	128/104	136

Pressure variations in mm Hg and duration in seconds for the single pressure pulse phases given for 20 pressure pulses recorded from 10 different dogs during rest.

constant for the individual animal. On the other hand the absolute pressure fall is greatest when the pulse period is most prolonged. Between the other values noticed above and the pulse-rate and blood-pressure no correlation can be established. The amplitude and period of the diastolic pressure wave cannot be calculated and judging from a rough estimate there is no relation between its amplitude and blood-pressure or pulse-rate. The pre and post oscillations (a, c) are often faintly indicated on the pressure curve, but the initial oscillation (b) very often cannot be detected. They have as a rule the same frequency (30—50 Hz). The amplitude is very small in relation to the thickness of the lines of the pressure curves and as the oscillations do not take place about a constant pressure level but are superimposed upon the main course of the pressure curve, an exact calculation of their damping is impracticable.

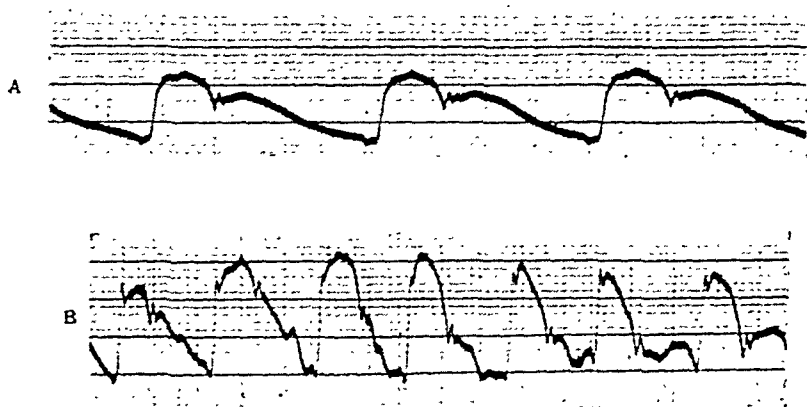
Both the observed absolute pressure values and the form of the pressure curve are in agreement with the results previously obtained in unanaesthetised animals (*Parkins* 1934, *Gregg et al.* 1937, *Hamilton et al.* 1938, 1940), nor are there any deviations from the pressure course observed by earlier authors on narcotised animals (Fig. 1). In pressure curves recorded just after the insertion of the catheter the diastolic pressure wave is missing (*Skouby* 1945). In these recordings and in those first discussed, where the diastolic wave was absent, the pressure fall CE made up an insignificant part of the pressure fall in diastole.



## CHAPTER VI

### THE SHAPE OF THE AORTIC PRESSURE CURVE DURING WORK

The pressure courses at rest and during work differ from each other on many points (Figs. 18, 19 and 20). When work begins the initial steep systolic pressure rise AB increases in gradient and height. The following slower rise BC decreases as a rule and often the pressure maximum is reached in the first phase, so that the phase BC vanishes. The end of systole is distinctly marked in most of the resting curves. In the working curves the last part of the systolic pressure course and proto-diastole cannot be distinguished. The pressure fall CE accounts for the greater part of the pressure fall in each cycle, in contrast to the relation during rest. If the amplitude is not increased



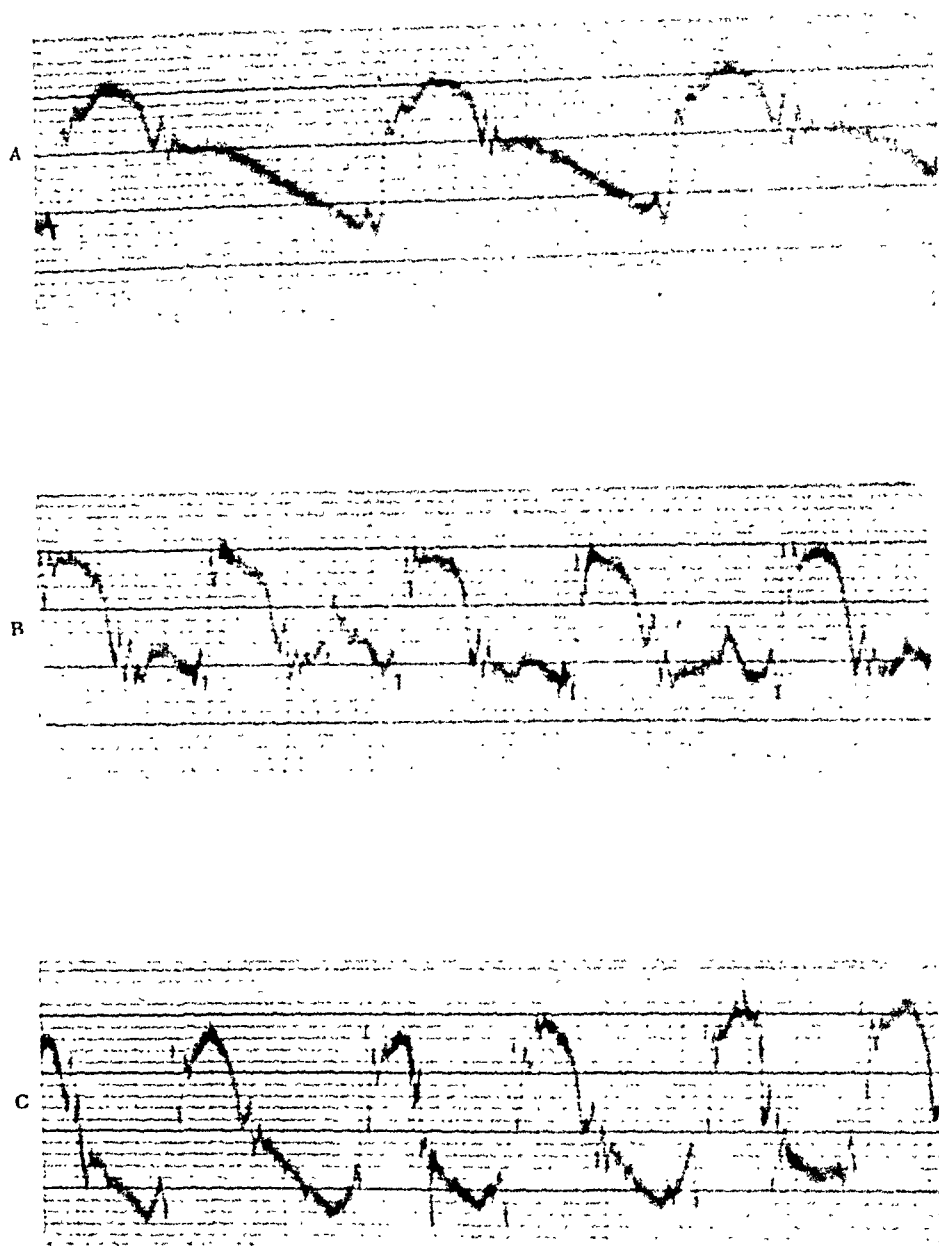
*Fig. 18.*

Aortic pressure curves in a dog under rest and during work.

A: Rest. Blood-pressure 133/97. Pulse-rate 143.

B: 1.5 minutes after the beginning of work. Blood-pressure 154/107.  
Pulse-rate 242.

Time mark 1/50 & 1/10 second.



*Fig. 19.*

Aortic pressure curves from a dog before and during work.

A: Before work	.....	Blood-pressure 193/159	Pulse-rate 157
B: 11 seconds after the beginning of work	.....	208/168	225
C: 17	.....	220/166	230

Time mark 1/50 & 1/10 second.

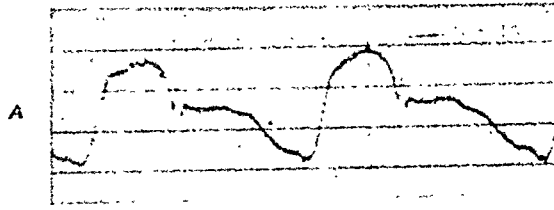
significantly, the diastolic pressure course may become nearly horizontal. Where the amplitude is increased substantially, the pressure decreases in diastole and as great a fall in pressure as at rest may occur. The positive diastolic pressure wave is flattened or disappears and the secondary oscillations as a rule increase in amplitude. The quantitative changes in a single experiment (Fig. 20) are seen from table 13, where the duration of the individual pressure phases, and the onset of the pressure variations in them are quoted for the dog at rest, during part of the transition from rest to work, and for short periods after work has continued for 1,5 and 6,5 minutes. In this experiment the pressure rise AB changes from 40 mm to a maximum of 65 mm Hg. and the rate of pressure rise increases from 1 mm/ms to 2,4 mm/ms. The ratio of the pressure fall before the closure of the semilunar valves to the amplitude, which during rest is less than 0,5, is always greater during work, reaching a maximum of 0,9. The changes set in before the blood-pressure alters, and both the gradient and the absolute pressure increase in AB vary directly as the pulse-rate. The other changes seem to be independent of the blood-pressure level and pulse-rate.

Besides the pressure changes referred to, other changes may occur very inconstantly in the pressure course due to pressure components produced by movements of the dog or by the distal part of the catheter sometimes pushing against the wall of the aorta. They are not found on the horizontal line recorded when

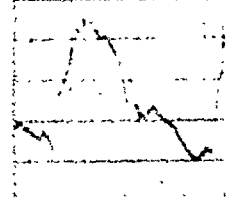
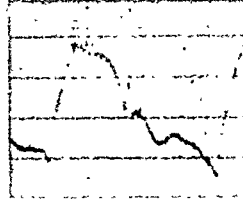
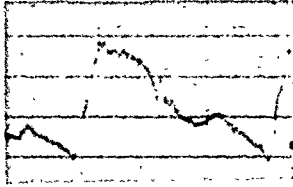
Fig. 20.

Pressure pulse curves from the aorta before and during work.

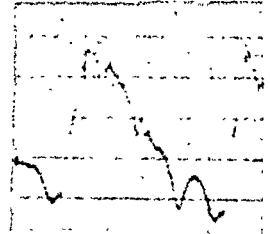
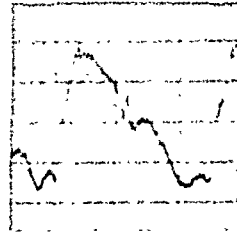
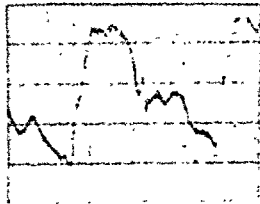
A: Before work .....	Blod-pressure	180/130	Pulse-rate	160
B: 4 seconds after the beginning of work	»	182/130	»	207
B: 6   »       »       »       »       »	»	184/124	»	240
D: 7   »       »       »       »       »	»	194/130	»	250
E: 8   »       »       »       »       »	»	194/138	»	260
F: 9   »       »       »       »       »	»	198/136	»	250
G: 10   »       »       »       »       »	»	196/120	»	240
H: 1,5 minutes   »       »       »       »	»	193/130	»	237
I: 6,5   »       »       »       »       »	»	224/156	»	254



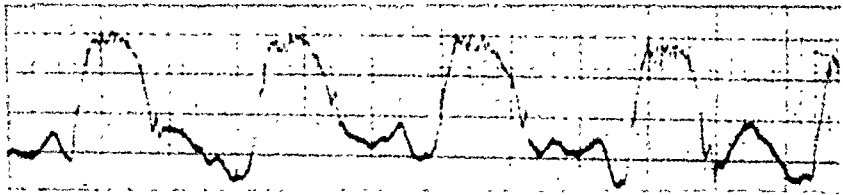
B, C, D



E, F, G



H



I

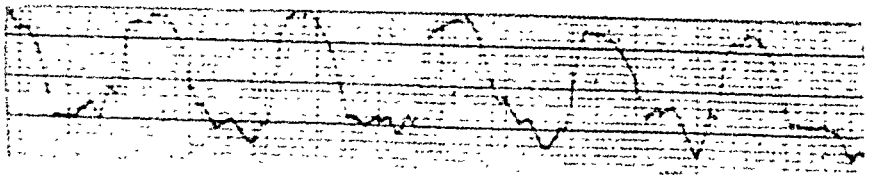


Fig. 20.

Duration of work	AB	AB	BC	BC	CD	CD	DE	DE	EA	EA	Blood pressure	Pulse-rate
	Pressure alteration in mm Hg.	Duration in seconds	Pressure alteration in mm Hg.	Duration in seconds	Pressure alteration in mm Hg.	Duration in seconds	Pressure alteration in mm Hg.	Duration in seconds	Pressure alteration in mm Hg.	Duration in seconds		
Rest .....	40	0.04	4	0.05	4	0.03	16	0.02	28	0.21	178/134	177
	44	0.04	12	0.06	12	0.04	12	0.01	34	0.22	186/130	167
	36	0.04	6	0.05	6	0.03	15	0.02	35	0.23	182/176	162
4 Sec. ....	52	0.04	0	0	14	0.06	14	0.015	24	0.16	182/30	207
6 Sec. ....	54	0.04				CF	CE					
7. Sec. ....	56	0.05				30	0.08		30	0.14	184/124	240
8 Sec. ....	62	0.035	2	0.03		47	0.06		23	0.14	194/130	250
9 Sec. ....	62	0.04	0	0		36	0.05		20	0.13	194/138	260
10 Sec. ....	66	0.04	0	0		40	0.07		22	0.14	198/136	250
						38	0.08		38	0.15	196/120	240
1½ Min. ....	52	0.035	2	0.03		41	0.055		20	0.13	194/133	240
"	59	0.050	3	0.03		38	0.060		12	0.14	194/144	222
"	50	0.040	0	0.04		41	0.055		20	0.15	194/133	214
"	57	0.035	0	0.04		52	0.045		5	0.15	190/133	222
6½ Min. ....	60	0.04	1	0.03		36	0.06		24	0.12	224/163	250
"	65	0.04	0	0.03		41	0.05		20	0.13	228/167	230
"	60	0.025	5	0.06		35	0.04		30	0.10	228/163	270
"	57	0.035	0			30	0.085		27	0.10	220/159	270

Pressure alterations in mm Hg and duration in seconds for the single phases of pressure pulse curves recorded from a single animal at different times after the beginning of work.

the connection between the aorta and the condenser manometer is interrupted, and cannot therefore be attributed to tremor of the recording system. By surveying a series of consecutive pressure pulses, one can usually determine what changes are due to real blood-pressure fluctuations and which are due to extraneous disturbances.

## CHAPTER VII

### THE SHAPE OF THE AORTIC PRESSURE CURVE UNDER THE INFLUENCE OF DRUGS ACTING ON THE CIRCULATION

#### *Amyl nitrite.*

Inhalation of amyl nitrite produces a peripheral vasodilatation (*Schram* 1875, *Cash and Dunstan* 1893). In man a fall in blood-pressure of 11—14 per cent occurs (*Wallace and Ringer* 1909), while the blood-pressure in dogs most often falls slightly or rises. This reaction is attributable to the reflex rise of pulse-rate in this animal being often very pronounced, so that a compensation for the vasodilatation occurs (*Brunton* 1871). The pure action of nitrites is seen only with tracheal inhalation, as nasal inhalation of the drug releases disturbing reflexes from the mucous membrane of the nose (*Filehne* 1874).

#### *Technique.*

In the present investigations amyl nitrite is inhaled through nose and mouth, as it was considered inexpedient to make a tracheotomy in addition to the insertion of the catheter required for the pressure recordings. 10 experiments have been performed. In 3 of them pure fluid amyl nitrite was used and in the others a 20 per cent solution in alcohol and ether, as it was impossible to procure a sufficient amount of the pure drug. The mode of exhibition was in all cases the same, i. e. a cotton-wool tampon soaked in the fluid was placed in an anaesthetic mask, through which the inspired air was made to pass. While inhalation of the pure drug produced a transient excitation this was not the case when the solution was used.

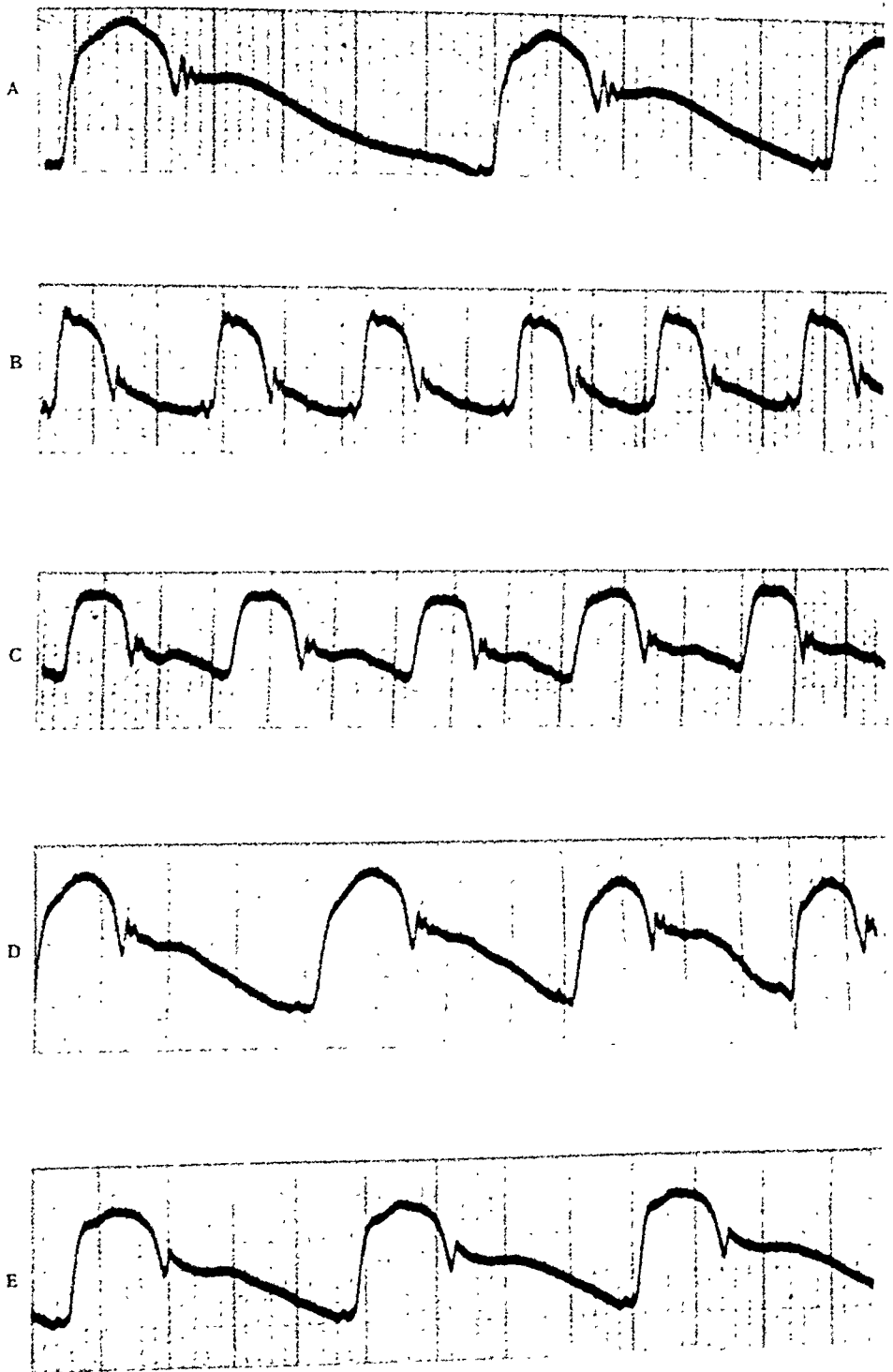
*Results.*

In all experiments the blood-pressure remained, apart from minor oscillations, at the initial level or rose 10—30 mm. In the experiments using amyl nitrite solution a periodical reduction in pulse-rate resulted, but the pressure course never varied appreciably from the pressure curves found during rest. In 2 of the experiments using pure amyl nitrite, distinct changes in the pressure course were produced, but not in the 3rd experiment. The nature of the changes produced is seen in Fig. 21, which shows the pressure course at different times during the experiment when the changes were most distinct. The blood-pressure in this experiment rose from 166/116 to a maximum of 198/169 and the pulse-rate, which before the inhalation was about 100, rose to a maximum of 300 in the first minute after the inhalation began. The animal was to begin with very agitated, but when, after 15 seconds the inhalation was discontinued, the dog lay down on its side and remained quiet during the remainder of the experiment. In spite of this the pulse-rate 3 minutes after the inhalation ceased was 250. At that time the rise AB constituted the whole of the systolic rise. The rate of pressure increase, 1,5 mm/ms at rest, rose to 1,75 mm/ms, but the pressure amplitude was unchanged. The pressure fall from the systolic maximum to the end of protodiastole, which at rest was about half of the amplitude, now amounted to 82 per cent of this. The diastolic pressure course was flattened and the pressure fall in this period was diminished. The amplitude of the secondary oscillations increased.

*Nitroglycerine.*

The action of nitroglycerine is in the main the same as that of amyl nitrite but weaker. 4 experiments have been performed with a dosage of 0,5—2 mg nitroglycerine by mouth in tablet form. Fig. 22 shows the changes in the pressure course under its influence in one experiment. The blood-pressure of the experimental animal was before the action of the drug the highest measured in all investigations (216/160) and the pulse-rate 110. During the experiment the pressure fell to 180/130 without any



*Fig. 21.*

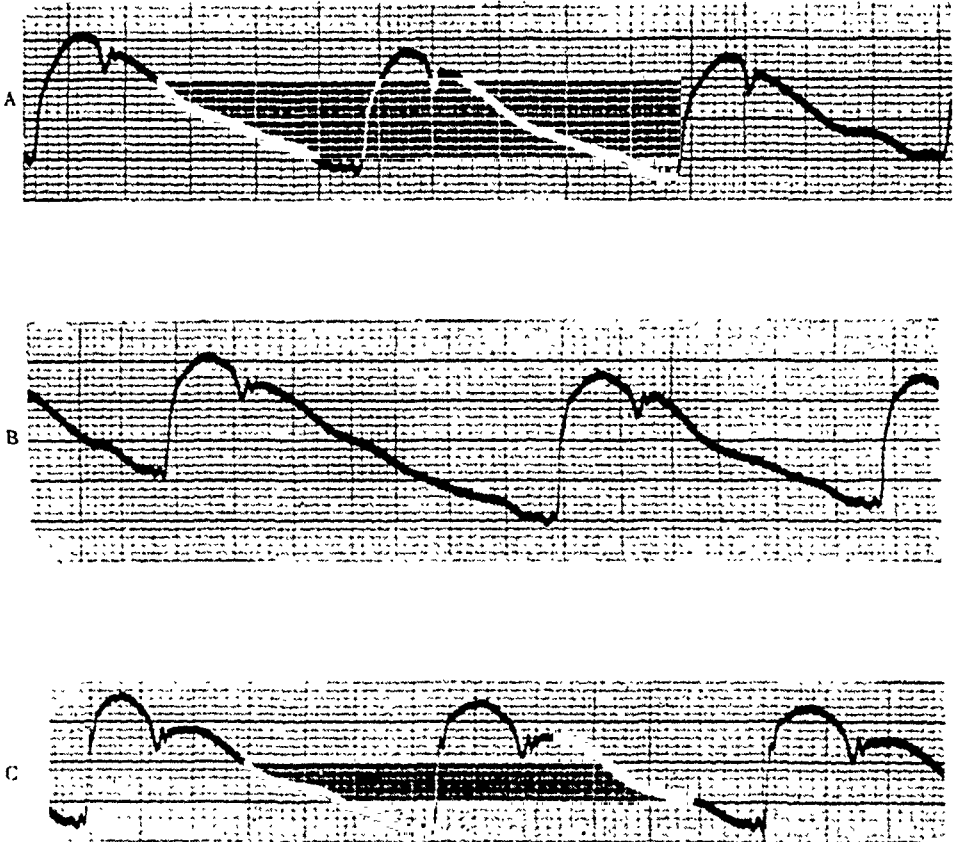


Fig. 22.

Pressure pulse curves from the aorta before and during the action of nitroglycerine.

A: Before intake of nitroglycerine	....	Blood-pressure	213/161	Pulse-rate	120
B: $\frac{3}{4}$ minute after intake of nitroglycerine			194/146		85
C: $4\frac{1}{4}$ " " " " " "			179/130		99

Time mark 1/50 & 1/10 second.

Fig. 21.

Pressure pulse curves from the aorta before and during amyl nitrite action.

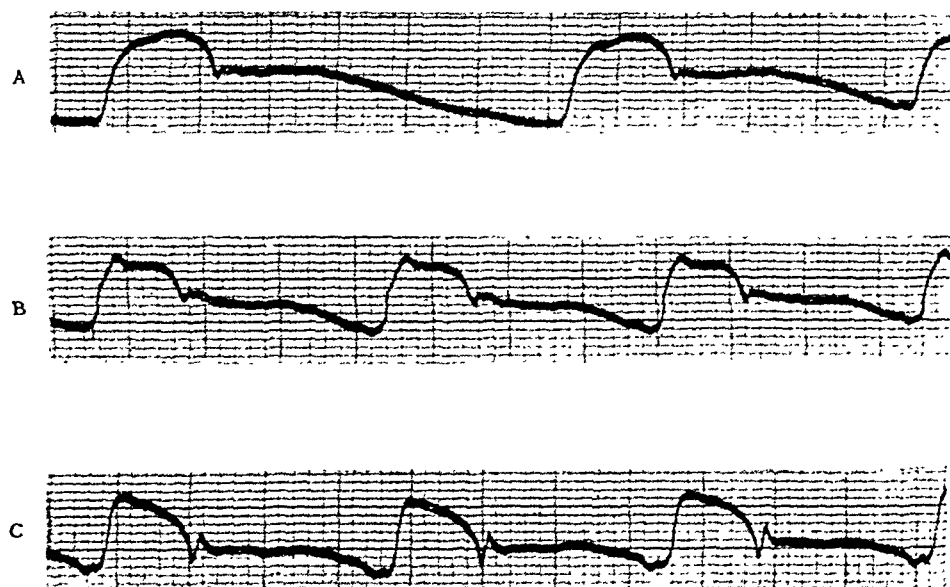
A: Before the action	.....	Blood-pressure	166/116	Pulse-rate	113
B: 3 minutes after the beginning of the action			186/142		250
C: 5 " " " " " "			198/167		202
D: 8 " " " " " "			192/141		150
E: 10 " " " " " "			187/136		127

Time mark 1/50 & 1/10 second.

change in pulse-rate. The steep systolic rise AB increased from 34 mm to a maximum of 42 mm, with a rate of rise of 2 mm/ms (ca. .1 mm/ms during rest). The pressure rise BC, 20—30 mm at rest, was reduced to 6 mm, the fall in the period CD was increased from a resting value of 0—3 mm to 6 mm, while the fall DE was unchanged (ca. 10 mm). In this way the pressure course in BD developed a more rounded form. The positive diastolic pressure wave, not very marked before the experiment, became more and more conspicuous as the pressure fell. The initial oscillations were pronounced during the whole experiment. Pre- and post-oscillations were accentuated and were most pronounced at the low pressure values.

### *Histamine.*

Injection of histamine into dogs produces a dilatation of capillaries and of the smallest peripheral arteries, and larger



*Fig. 23.*

Pressure pulse curves from the aorta before and during the action of histamine.

A: Before the action .....	Blood-pressure	166/130	pulse-rate	96
B: 1 minute after the injection of $\text{cm}^3$ histamine	,	166/135	,	163
C: 5.5 , , , , , , ,	,	121/89	,	156
Time mark 1/50 & 1/10 second. .				

doses induce a circulatory failure of peripheral origin (*Dale and Laidlaw* 1910, *Dale and Richards* 1918, *Burn and Dale* 1926). 8 experiments have been performed with a dosage of 1—7 mg histamine hydrochloride (Merck) by subcutaneous injection. The observed changes in the pressure course were uniform from experiment to experiment and appear in fig. 23, which shows pressure curves recorded at different moments in a single experiment. The blood-pressure at rest was 166/130 mm and the pulse-rate 100. 5 minutes after the action of the drug began the pressure was 121/89 and the pulse-rate 155. The pressure rise AB, 36 mm at rest, decreased to 32 mm Hg. At the same time the rate of rise increased in this phase 0,6 to 1 mm/ms. The phase BC disappears and the distinction between systole and protodiastole, very clear at rest, is wiped out. The pressure fall CE increases from 15 to 28 mm and makes up 88 per cent of the amplitude, which is reduced from 39 to 32 mm. The diastolic pressure course becomes nearly horizontal, as the positive pressure wave in this phase is flattened. The pre- and post-oscillations are accentuated.

### *Acetylcholine.*

Acetylcholine (*Baeyer* 1867, *Hunt and Taveau* 1906, *Loewi* 1921) produces a vasodilatation and by intravenous injection causes a fall in blood-pressure even in very small doses (*Dale and Dudley* 1929, *Feldberg and Krayner* 1933, *Zinnitz* 1938).

Three experiments have been performed with the exhibition of very large doses of acetylcholine Hoffmann La Roche (10 cg) intravenously. In all experiments a marked fall in blood-pressure occurred, so that the pressure course is changed, as seen in Fig. 24. In this experiment the pressure fell in the first minute from 140/84 to 92/44, with no change in pulse-rate (130—133). In the phase AB the rate of rise was reduced from 1,2 mm/ms to 0,8 mm/ms and under the influence of the drug the whole systolic pressure rise occurs in this period. The pressure fall, which before the exhibition of the drug was about the same in the periods CE and EA, now occurs almost exclusively before the closure of the semilunar valves (96 per cent), so that the diastolic pressure course becomes horizontal. 4 minutes after

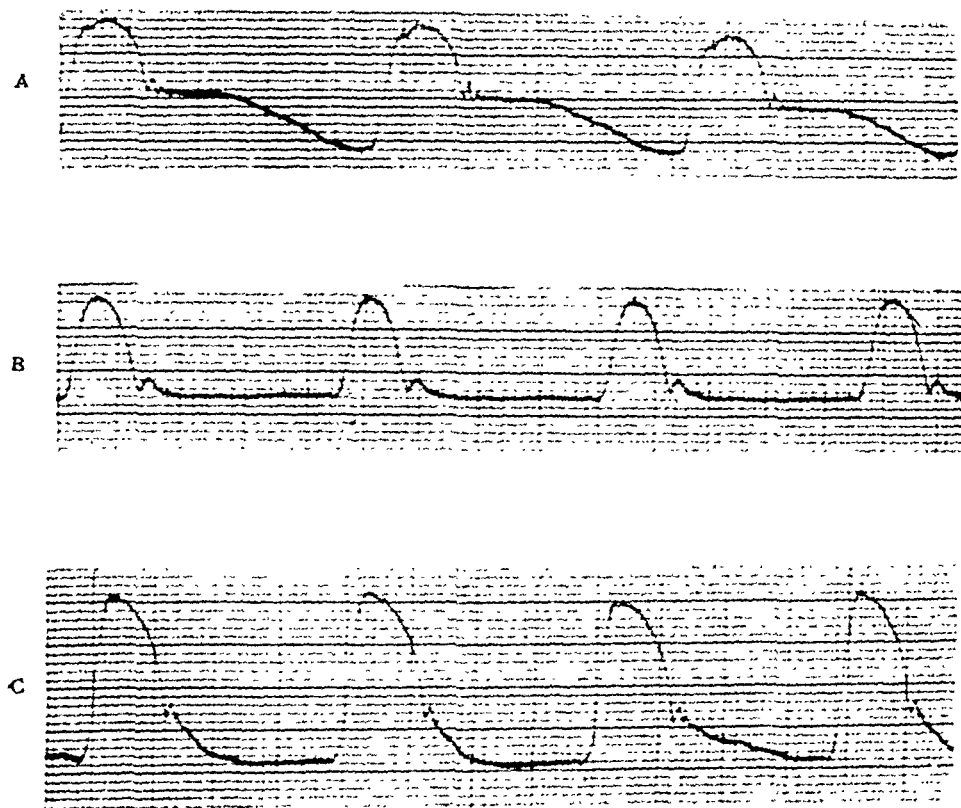


Fig. 24.

Pressure pulse curves before and during the action of acetylcholine.

A: Before the action .....	Blood-pressure 140/83	Pulse-rate 130
B: 1.25 minute after the beginning of the action >	92/42	> 133
C: 3.75 > > > > > > >	140/64	> 150

Time mark 1/50 & 1/10 second.

the injection the pressure was 140/60 and the pulse-rate ca. 150. The amplitude had then risen to 76 mm and the rise in phase AB was 1.9 mm/ms, while CE alone accounts for 80 per cent of the total pressure fall in the cycle. The pre-oscillations disappear and the frequency of the post-oscillations is diminished but cannot be measured.

### *Adrenalin.*

Adrenalin produces a powerful contraction of some arteries, capillaries and veins, especially in the splanchnic area (*Dale and Richards* 1918). The action on the circulation is manifested

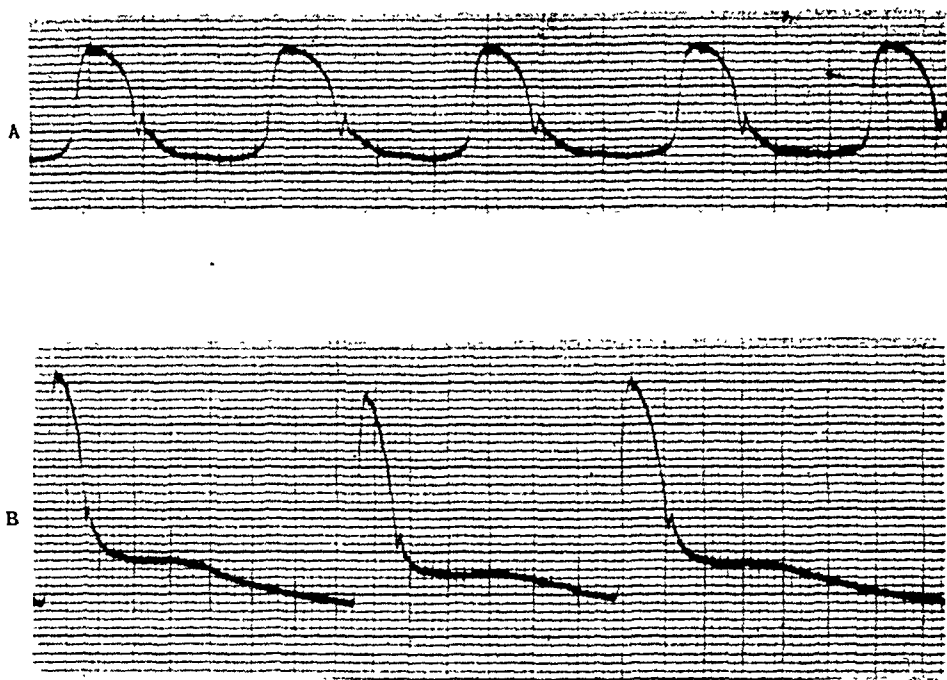


Fig. 25.

Aortic pressure curve under influence of acetylcholine and adrenaline.

A: Pressure course during the action of acetylcholine.

Blood-pressure 125/70. Pulse-rate 171.

B: The course just after intravenous injection of 1 mg adrenaline hydrochloride.

Blood-pressure 176/72. Pulse-rate 90.

Time mark 1/50 & 1/10 second.

most often as a rise in blood-pressure (*Oliver and Schäfer* 1894), followed by a decrease in pulse rate. It has been shown in man that the cardiac output increases after subcutaneous injection of the drug (*Liljestrand and Euler* 1929). Intravenous injection of very small amounts of adrenaline in unanaesthetised dogs produces a relatively constant pressure fall, affecting chiefly the systolic values (*H. C. Wiggers et al.* 1942).

Four experiments have been performed with injections of 0.5—2 mg adrenaline hydrochloride intramuscularly. The pressure rose about 20 per cent in 3 of the experiments and decreased about 10 per cent in the 4th. No change in the form of the pressure curve could be demonstrated. In one animal, where the pressure was reduced from 139/84 to 125/70 after injection of acetyl-

choline and the form of the curve changed in the manner above described, the intravenous injection of 1 mg adrenaline hydrochloride augmented the rise AB from 55 mm to 105 mm Hg. without prolongation of this phase. The pressure fall in CE was changed from 80 to 70 per cent of the total pressure fall. Simultaneously the pulse-rate decreased from 170 to 90. The diastolic pressure course trended towards normal values, with a distinct positive pressure wave (Fig. 25).

Summing up, the investigations show that under the influence of vasodilator drugs the pressure rise AB makes up the greater part of the amplitude, and the rate of rise in this period increases, with the blood-pressure fall becoming less pronounced. In such case AB decreases in gradient and height in the same manner as in narcotised animals, when the venous return to the heart is diminished or anoxaemia is produced (*Wiggers* 1918, *Sands and De Graff* 1925). The pressure fall CE increases both when the pulse-rate increases and when there is a significant fall in blood-pressure, so that the pressure fall after the closure of the semilunar valves decreases, which accords with the results earlier obtained in narcotised animals under the influence of vasodilator drugs (*Frank* 1925/26, *Wiggers* 1915) shock (*Wiggers* 1918) or anoxaemia (*Sands and De Graff* 1925). The changes observed under the influence of adrenaline are similar to the changes in narcotised animals suffering from shock, when the abdominal aorta is compressed (*Katz and Wiggers* 1927).

## CHAPTER VIII

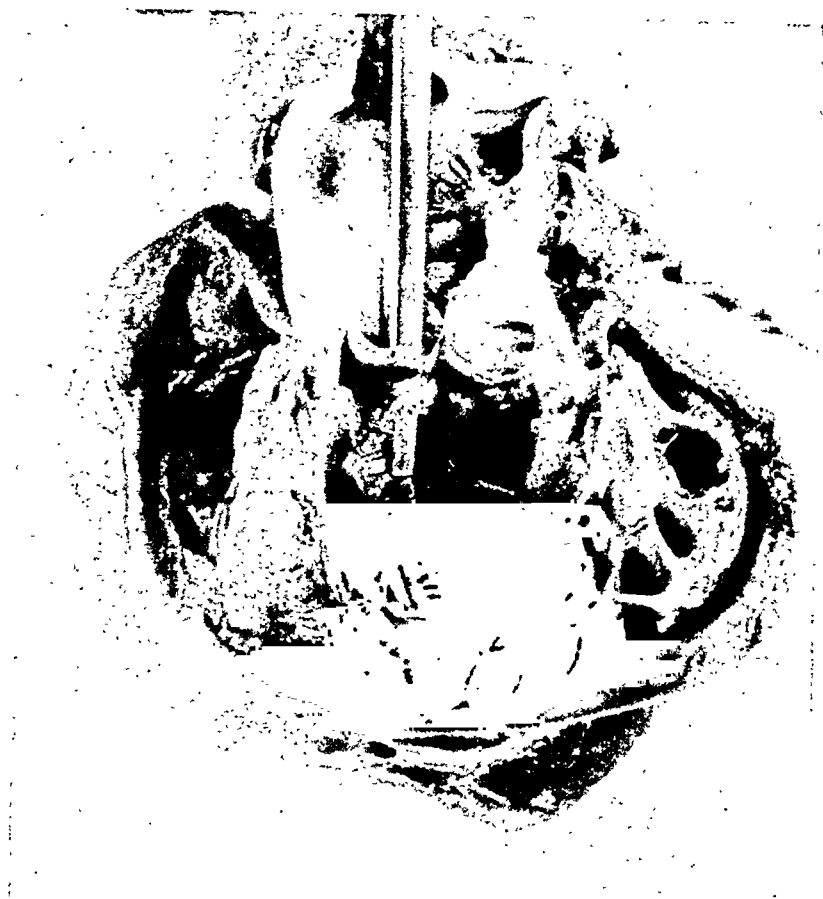
### THE SHAPE OF THE AORTIC PRESSURE CURVE IN AORTIC INSUFFICIENCY

The pressure recording is carried out in the usual way and the insufficiency is produced by perforating a semilunar cusp with a long stylet introduced through the catheter. The lesion was produced in only one animal, which remained without symptoms of circulatory insufficiency in the first 24 hours after the injury. In the next 24 hours it became strongly dyspnoeic with irregular (coupled) pulse. When the blood-pressure level became substantially reduced and the dog could only with difficulty move a few steps at a time, it was killed after the pressure-pulse curves had been recorded. The autopsy showed a nearly circular perforation with ragged edges and a diameter of 4—5 mm in one semilunar valve (Fig. 26).

#### *Results.*

After the operative interference both the course of the aortic pressure and the absolute pressure values were changed. By comparison with the course prior to the production of the insufficiency (Fig. 27), the pressure amplitude is seen to be substantially increased, from 140/105 to 160/100. The increase in amplitude is therefore due to an increase in the systolic blood-pressure and only in lesser degree to a decrease in the diastolic. 48 hours after the injury a lowering of both the systolic and diastolic pressures is observed, to 118/50—40. The initial systolic rise AB increases in height, from ca. 30 mm Hg. to 35—50 mm and the rate of rise increases from ca. 0,7 mm/ms to 0,9—1,3 mm/ms. The succeeding rise BC increases from 4—5 mm Hg. to 15—30 mm Hg. and amounts to 40 per cent of the total systolic pressure rise against 14—16 per cent before the





*Fig. 26.*

The opened heart of the dog with experimentally produced aortic insufficiency.  
The probe passes through the perforation.

---

*Fig. 27.*

The aortic pressure course before and after experimentally produced aortic insufficiency.

A: Before the insufficiency.

B & C: Pressure pulse curves recorded with an hours interval 24 hours after the production of the insufficiency.

D: Recordings 48 hours after the production of the insufficiency.

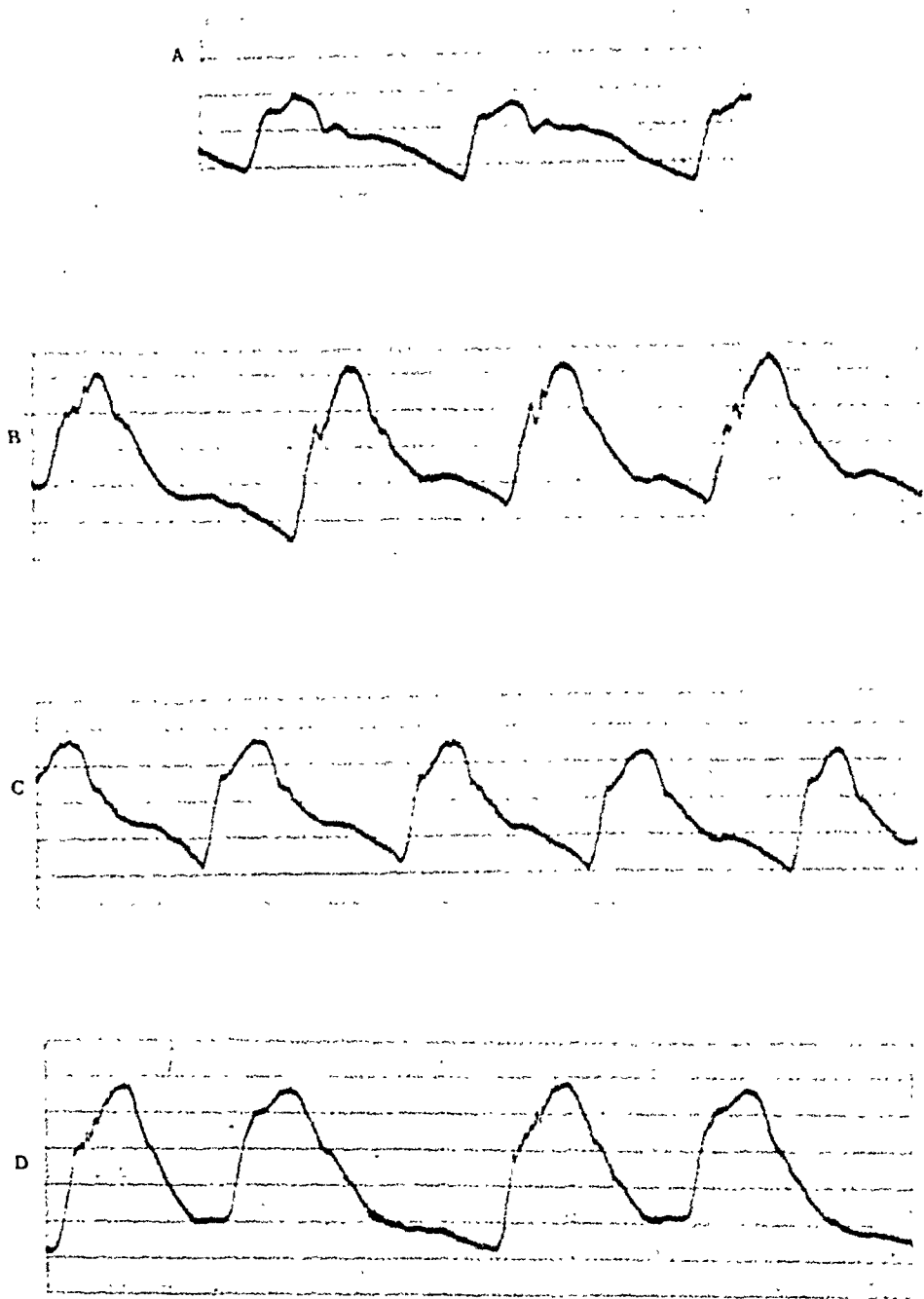
A: Blood-pressure 141/105      Pulse-rate 160.

B:                              159/91                              167.

C:                              161/102                              182.

D:                              118/50—40                              154—214.

Time mark 1/50 & 1/10 second.

*Fig. 27.*

insufficiency. The boundary between the end of systole and protodiastole is not marked in the pressure curve. In the two phases together the pressure falls ca. 20 mm, but their part in the total pressure fall in a cycle is unchanged. The end of protodiastole is often only faintly indicated by single slightly accentuated secondary oscillations. The diastolic pressure course differs from that of the normal animal in so far as a very considerable part of the pressure fall occurs in the first part of the period. 48 hours after the production of the insufficiency the mean pressure is low and nearly all the diastolic pressure fall occurs early in diastole, so that the pressure decrease in the remaining part is minimal, just as is seen during work and in circulatory insufficiency induced by vasodilator drugs. The pressure course 48 hours after the onset of the insufficiency shows further a bigeminal pulse, every other pressure pulse arriving very early in the preceding diastole. The first of the coupled pulse beats shows pressure waves superimposed upon the more uniform systolic rise, in part of very high frequency, and the steep systolic rise presents several nodes. The waves superimposed upon the descending part of the pressure curve have a much lower frequency than the former (Fig. 27).

*Wiggers'* explanation of the characteristic change in the pressure pulse shape is "that an aortic regurgitation increases the initial intraventricular tension, owing to a regurgitation of pressure during diastole; this in turn causes a more vigorous ejection of a larger blood volume in the early portion of the next systole", and not, as had been earlier supposed, a reflex dilatation of the peripheral vessels (*Steward* 1908). *Wiggers* supports his assumption by the facts that the shape of the curve is accentuated under the influence of adrenaline, when the peripheral arteries are constricted, that aortic insufficiency induced by the action of nitroglycerine seems to increase the amplitude, and that the greatest fall in pressure occurred in diastole. That the results of previous authors supported the assumption of vasodilatation as the cause he attributed to the impairment of the circulation by extensive interference in the production of the aortic insufficiency. His own experiments are not free from this criticism, however, as they too were preceded by severe surgical

interference, involving pronounced changes in the shape of the curve, before the insufficiency was produced. To ensure that the mean pressure did not fall too much he had to compress the aorta. The experiments in this work show that the systolic part of the aortic pressure curve in aortic insufficiency has a close resemblance to the course of the ventricular pressure in the same period in heart-lung preparations with valvular function intact, when the diastolic inflow is increased (*Patterson, Piper and Starling* 1914, *Straub* 1926). The course also corresponds to the systolic part of the aortic pressure curve in the anaesthetised animal with good diastolic filling (*Wiggers* 1928). The part of the pressure curve in which the pressure is falling corresponds to the results obtained in the present work for the course during work and under the influence of vasodilator drugs. The pressure course in its intirety can therefore be explained by Wiggers' assumption, that the pressure in diastole is transmitted to the ventricle, thereby increasing the degree of tension of the muscle, so that the contraction occurs with greater force than before, in the same way as when the residual blood volume increases. The pressure in the aortic root falls rapidly, due to a diastolic regurgitation, besides the energy transmission to the peripheral vessels, and the incisura is diminished because only a part of the kinetic energy directed towards the heart is reflected while the remainder is transformed into potential energy consumed in increasing the degree of tension of the ventricular musculature.

It is in agreement with the changes in the circulatory function assumed above, that the increase in amplitude is chiefly effected by an increase of the systolic blood-pressure so long as the heart can respond to the increased load. That Wiggers found the cause was chiefly a reduced diastolic pressure may be attributed to the fact that his experiments were performed on narcotised animals and involved such extensive operative interference that this in itself produced essential changes in the pressure course, similar to those in shock and impaired cardiac function.

In man with aortic insufficiency it is sometimes observed that the indirectly measured diastolic pressure falls to zero. As this is not the case in the present experiment when circulatory

insufficiency was produced, it may be assumed that the reason for the different results is that, just as in the working experiments where the pressure fall also occurs rapidly, the indirectly measured diastolic blood-pressure is not a true expression of the blood-pressure at the close of diastole.

### *The action of nitroglycerine upon aortic insufficiency*

is investigated by the exhibition of 1 mg of the drug per os. 2 experiments have been performed an hour apart 24 hours after the onset of the insufficiency. In both experiments there resulted, after a transient fall in both systolic and diastolic pressures, an increase in pulse-rate and a rise in pressure above the initial level. This rise was succeeded by a fall, which in one experiment ceased at the initial level, but in the other was somewhat more profound. The amplitude remained unchanged in both experiments. Fig. 28 shows the changes in the shape of the pressure pulse. Both where the pulse is high and where the mean pressure is low the initial systolic rise is increased at the expense of the phase BC. Change in the diastolic course is always seen with low mean pressure and corresponds to the pressure course 48 hours after the perforation, where the blood-pressure was low without external influence. A similar course is also seen in the one experiment with raised blood-pressure associated with rapid pulse-rate, while this condition in the other experiment (Fig. 29) produced a less steep fall in diastole than before. The latter course corresponds approximately to the variations which according to *Wiggers* (1915) are typical of the action of nitroglycerine on narcotised dogs with aortic insufficiency, as recorded from the subclavian artery. The

*Fig. 28.*

Pressure pulse curves from a dog with experimentally produced aortic insufficiency before and during the action of nitroglycerine.

A: Before the action .....	Blood-pressure 159/91	Pulse-rate 167
B: 1 minute after intake of 1 mg nitroglycerine	131/68	174
C: 5 " " " " " " " " " "	164/100	160

Time mark 1/50 & 1/10<sup>2</sup> second.

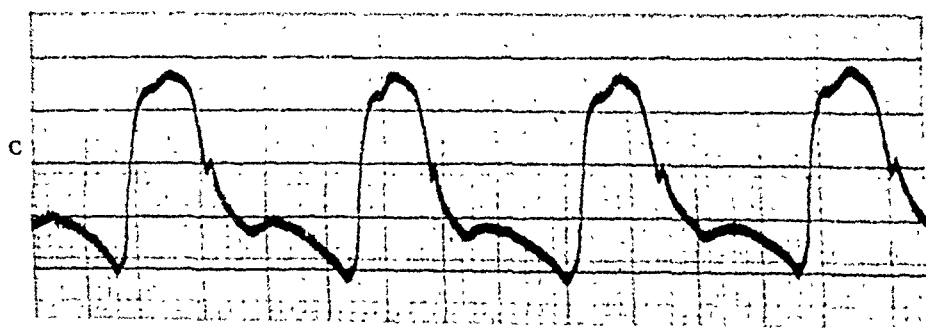
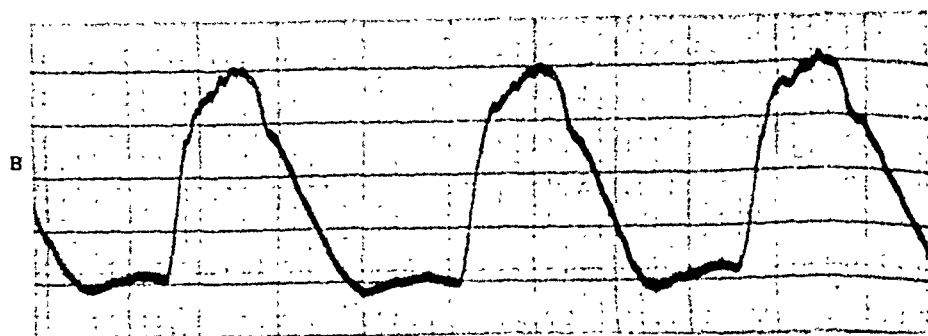
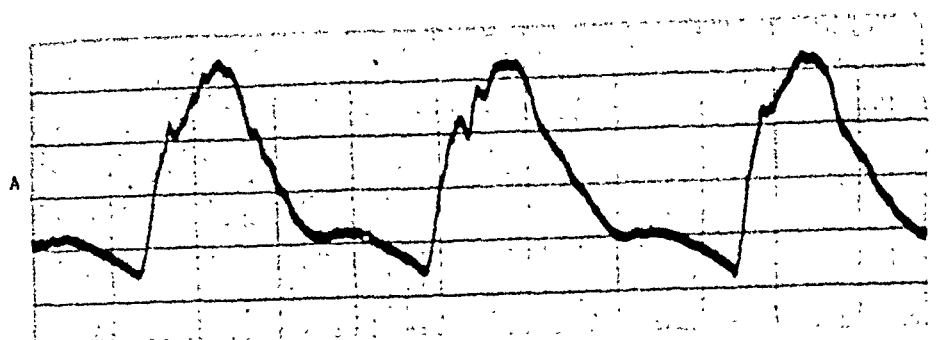
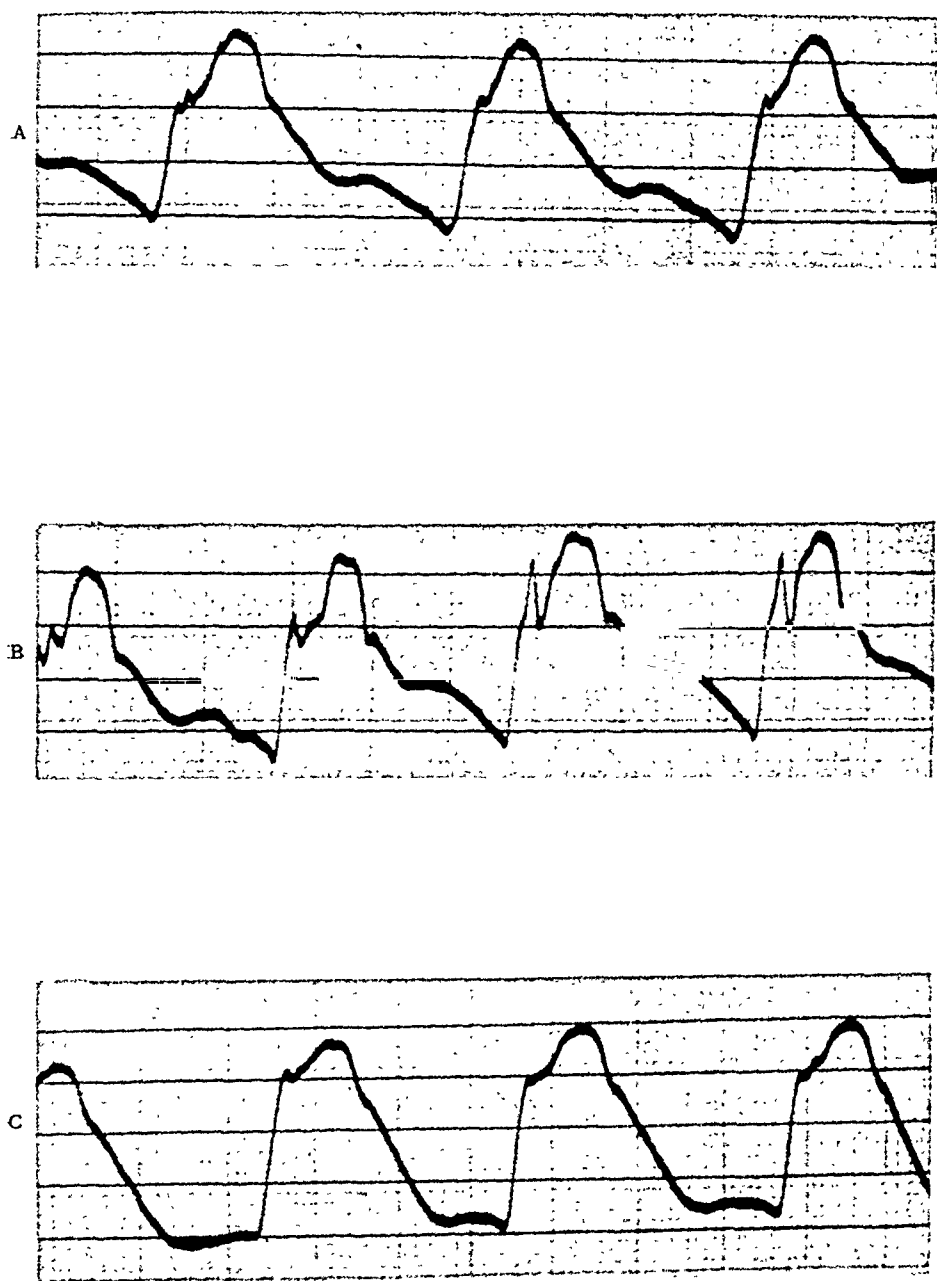


Fig. 28.

*Fig. 29.*

incisura on the different curves is only faintly indicated and is most distinct, indeed, the steeper the pressure fall is after the end of systole.

The observed changes are analogous to the pressure changes found in animals with intact valvular function under the influence of nitroglycerine. The reason why the incisura and the post-oscillations appear most distinct when the pressure changes are most rapid may be sought in the fact that the ventricular pressure falls more rapidly after the end of systole under the influence of nitroglycerine and hence the pressure difference between the ventricle and the root of the aorta is increased, so that the regurgitation occurs with greater speed.

Fig. 29.

Pressure pulse curves from a dog with experimentally produced aortic insufficiency before and during the action of nitroglycerine.

A: Before the action .....	Blood-pressure 161/102	Pulse-rate 182
B: 1,5 minutes after intake of 1 mg nitroglycerine by mouth .....	180/120	207
C: 3 minutes after intake of 1 mg nitroglycerine by mouth .....	138/80	187
Time mark 1/50 & 1/10 second.		



## CHAPTER IX

### THE SHAPE OF THE PRESSURE CURVE IN THE AORTA UNDER VARYING CONDITIONS

From the investigations already discussed it appears that the aortic pressure changes its course in response to work, to drugs acting upon the circulation, and to aortic insufficiency. The changes observed during work for the first phase (AB) of the pressure curve correspond to an increased diastolic inflow, irrespective of whether this is produced artificially in heart-lung preparations or by regurgitation in aortic insufficiency. After the systolic maximum is reached the pressure curve during work corresponds to the conditions seen in response to the action of amyl nitrite and other vasodilator drugs. It is therefore natural to suppose that the left ventricle during work empties itself more rapidly than during rest, leading eventually to an increased output together with a diminished peripheral resistance.

The origin of the pressure pulses is due to energy produced by the contraction of the heart. In every systole the elastic vascular system is supplied with a certain amount of blood, whence arise pressure changes which disappear in diastole, at the end of which the pressure course shows an evenly declining pressure fall, granted that the next heart contraction does not occur prematurely. Only in such cases will the energy supply from one cycle affect the course of the next.

*Hamilton and Dow* (1939) have recorded the pressure course simultaneously from several places in the aorta in narcotised dogs and found that the course varied in a characteristic manner according to the site of measurement, in that the absolute value of the systolic pressure maximum increased towards the periphery. The interval between the beginning and the maxi-

imum of the pressure curve decreases until the distance from the heart to the site of recording exceeds a certain value, beyond which it remains unaltered. When this is the case a negative wave occurs at that place in the course where there is observed a positive diastolic wave in measurements in the root of the aorta. The incisura is wiped out, but the mean and diastolic pressures do not change. In experiments on a smaller dog with hypertension the position of the systolic maximum in the pressure pulse became constant at a lesser distance from the aortic root and occurred earlier in the pressure course than the systolic maximum in the aortic root. This must mean that the steepness in the phase AC increases. By simultaneously measuring the pressure course in the root of the aorta and at another more distal point in the aorta, the two pressure maxima may appear synchronously. A slight displacement of the measuring point caused the difference once more to appear. Later experiments were performed in which the descending aorta was occluded at different sites and the pressure was recorded just above these sites simultaneously with measurements at a series of points steadily decreasing in distance from the heart, by which means the authors observed the following changes in the pressure course in a given example:

1. The wave starts earlier.
2. A break develops in the anacrotic limb, which an accurate analysis of transmission times shows to be due to reflection at the point of occlusion.
3. The early diastolic waves do not show such a shift but remain in phase with those at the occlusion until a point 12,5 cm away is reached. Here they disappear, but 2,5 cm further back, at the top of the arch, they reappear exactly opposite in phase to those farther down. A node is thus clearly shown, a point of minimum pressure fluctuations with the waves on either side  $180^\circ$  out of phase or mutually reciprocal.

By occluding the aorta at different places it could be shown that the node shifted according to the length of the isolated segment. These results led *Hamilton and Dow* to suppose that

the shape of the pressure wave was due to the emptying of the heart originating by reflection a system of interfering pressure waves, which in turn interfere with the primary wave produced by the heart. The most important element in the conglomeration of secondary waves is a standing wave in the aorta. Later, (1944) *Hamilton* showed that the observed difference between the systolic pressure value in the carotid artery (considered as a central artery) and the femoral artery (considered as a peripheral artery) could be made to disappear on one side by injecting small amounts of acetylcholine in the femoral artery of that side. The vasodilatation produced by this means in the one extremity causes a local reduction of the peripheral resistance. During this time the systolic pressure course in the other femoral artery remains unchanged and the diastolic pressure continued unaltered on both sides. From this it can be seen that the high maximum in peripheral pressure curves is due to the resistance distal to the vessel area in question.

The conception of *Hamilton* that a standing wave is the essential element in the system of reflected waves in the aorta departs somewhat from the theory advanced by *von Recklinghausen*. According to the latter, local "Rückprallwellen" (rebound waves) arise by reflection of the primary wave produced by the contraction of the heart. The "Rückprallwellen" fuse together into a "swell" ("Gewoge"), which spreads to the separate vascular fields and rises so long as the energy supply exceeds that transformed into flow-energy, whereafter it decreases. In the central part of the arterial system the secondary waves occur only as the "swell" (das Gewoge), while "rebound waves" ("Rückprallwellen") become more pronounced as the pulse wave is recorded more distally. The "swell" (das Gewoge) is found throughout the arterial system, but its development differs according to the site, as it begins first nearest the heart and also reaches its maximum earliest here. For this reason "compensation" waves ("Gewogewellen") arise. The chief element in the conglomerate of secondary waves is thus an evenly rising and falling flood and not a standing wave. *Von Recklinghausen* draws attention to the fact that a certain interaction takes place between primary and secondary waves. The primary

wave wholly determines the secondary, but as the occurrence of the secondary oscillations has a bearing upon the pressure in the aortic root and so upon the resistance to the emptying of the heart, it must influence this emptying and thus also the primary wave.

The results obtained in the present investigation for the main course of the aortic pressure curve can all be explained upon the assumption that the primary centrifugal pressure wave interferes with a secondary pressure oscillation, the resultant of the reflected waves reaching the measuring point from the different vascular fields.

The differences in the pressure course under the varying experimental conditions can be explained by alterations in amplitudes, in the shape of the curves, or by a phase displacement. An increase of the systolic rise AB (change in amplitude) is seen in association with rapid pulse-rate during work and under the influence of vasodilator drugs when the blood-pressure level is not essentially lowered (Figs. 18, 19, 20, 22 and 24). Increase in amplitude occurs similarly in aortic insufficiency (Figs. 27—29). These changes are naturally attributed to an acceleration of emptying in the beginning of systole. A great height and rate of rise in the period BC (change in form and amplitude), as seen in aortic insufficiency (Figs. 27—29) and in hypertension (Fig. 22), can be ascribed to the secondary wave in this phase being strongly positive at the recording point and so summing with the primary. If the emptying is of short duration or the secondary wave is delayed, the primary wave will have passed its maximum before the secondary reaches the same phase as before (phase displacement) and hence a pronounced systolic and protodiastolic pressure fall appears (Figs. 18—24 and 27—29) and a conspicuous positive wave early in diastole (Fig. 22). If, at the same time, the peripheral resistance is diminished so much that the reflection capacity is weakened, the amplitude of the secondary wave is reduced (Figs. 18—24) and the diastolic pressure course becomes nearly linear. If the pressure fall before the closure of the semilunar valves is very pronounced the course in the following part of diastole becomes horizontal (Figs. 19, 20, 23, 24 and 25).

When several pressure maxima are found on the same pulse curve, the amplitude being reduced, it can be taken as an indication that they are produced by a damped oscillation (Fig. 22). The results do not conflict with *Hamilton's* conceptions. The more complicated theory of *v. Recklinghausen* explains only with difficulty how the maxima and minima superimposed upon the pressure curve occur without demonstrable phase displacement throughout the proximal part of the aorta (above the diaphragma), as seen in the pressure pulse curves of *Hamilton and Dow* (1939, Fig. 2). *Hamilton and Dow* hold the opinion that they have demonstrated the node of a standing wave. However, the observed alterations in the pressure course in the experiment upon which they base their conclusion (Fig. 2, *Hamilton and Dow* 1939) can be interpreted as arising from the displacement of 2 positive waves towards the end of diastole in successive recordings from the periphery towards the heart, from which it can be assumed that they are due to wave reflection. Further, all parts are not in phase in the one or the other end of the aorta. The results of *Hamilton and Dow* can therefore be equally well explained as arising by interference between the primary and reflected waves as by the occurrence of standing waves. Whichever is the case it must be accepted that the pulse wave is not a rhythmic wave motion passing over the arterial system from the heart to the periphery. It is built up of numerous components which vary in origin and strength from place to place depending upon the function of the heart and the elastic constants of the vascular system, which show marked differences in different sections of the vascular system. It is therefore impossible to gain information about the nature and transmission of the pulse wave by a Fourier analysis of central and peripheral pulse curves, as attempted by *Porjé* (1946).

## CHAPTER X

### REGULATION OF THE CIRCULATION DURING WORK

The rapidly occurring rise in pulse-rate after the beginning of muscular work must be released through nerve pathways. It has been shown in animal experiments that cutting of the parasympathetic filaments to the heart produces a marked rise in pulse-rate and that the pulse-rate rises inconsiderably during work afterwards, while removal of the sympathetic innervation is without consequence (*Samaan 1935, Essex et al. 1943*), so that the reaction in the last analysis is due to a lowering of vagus tone, which may arise in a variety of ways. The rise can scarcely be conditioned by psychical factors, since even persons much accustomed to the experimental routine show upon a sudden start an instantaneous rise in the previously measured pulse-rate, and the observed rises in the present animal experiments, where the start is passive and gradual, do not occur with the command to begin, but after the work has begun. The hypothesis has been put forward that the pulse is regulated by cortico-motor centres (*Johanson 1895, Krogh & Lindhard 1913*), but later investigations upon man (*Asmussen, Nielsen and Wieth-Pedersen 1943*) have clearly shown that the same rise in pulse-rate is reached in relation to the output of work, whether the work is electrically induced or performed in the usual way, so that other regulation mechanisms are possible for instance regulation through impulses from the working muscles. An increase in pulse-rate produced by augmented venous inflow (Bainbridge reflex) is hardly conceivable immediately after the beginning of work, but may perhaps be of significance in the later course, as the increased gradient and height of the steep systolic rise of the pressure curve is indicative of an increased cardiac output as the changes of the pressure course in the very

first seconds can be due solely to the left ventricle emptying itself in the main in the first part of systole and with greater force than before. Hence the volume of blood ejected in this period is increased and also the speed of the emptying.

It is improbable that the increase in pulse-rate with the beginning of work is released through an alteration in the impulses from the presso-sensitive zones, since the pulse-rate rises without a preceding fall in blood-pressure.

A few seconds after the beginning of work the blood-pressure rises in spite of the total peripheral resistance being barely increased. This happens after the pulse-rate has begun to rise and must be taken as an expression of the steady supply of more energy to the root of the aorta than disappears caused by the increased cardiac output. When the pressure begins to rise it must be assumed that the diastolic filling is increased so much that the cardiac output is also increased, since the amplitude grows excessively after the beginning of the pressure rise. This would hardly be the case if the degree of filling was unchanged or diminished, since the heart musculature would then contract with greater force despite constant or decreasing resting length.

The increased filling of the central veins must be ascribed to dilatation of the muscular vessels and contraction of the vessels of the portal area and of the organs not working (*Krogh* 1912, *Jarisch & Ludwig* 1927, *Barcroft* 1926, *Barcroft & Stephens* 1927). Perhaps the contraction of the skin vessels also plays a rôle in this connection (*Christensen & Nielsen* 1942). That the total peripheral resistance is already lowered early in the course of the work seems to emerge from the fact that the diastolic pressure course during work corresponds to the behaviour under the action of amyl nitrite, histamine and acetylcholine and to the diastolic course in aortic insufficiency. The nearly horizontal pressure course sometimes seen in diastole must be due to a very rapidly occurring state of equilibrium between the energy supplied to the measuring point from the dilated elastic arterial walls and the amount of energy which disappears as kinetic energy. It is possible that the presso-sensitive zones enter into the regulating mechanism which causes the blood-pressure during work in the "steady state" to remain at a constant level dependent

upon the rate of work. Such a regulation may arise from an alteration in sensitivity of higher centres caused by simultaneous impulses from other sources. It has been shown that the minute-volume during work in the "steady state" does not diminish even if half of the working muscles are excluded from the circulation by occlusion of the arterial supply, and that the same value is attained even if the occlusion had existed from the beginning of exercise (*Asmussen, Christensen and Nielsen 1940*). Later investigations (*Asmussen, Nielsen & Wieth-Pedersen 1943*) demonstrated that the minute-volume, pulse-rate and blood-pressure depend upon the rate of work, whether it was performed in the usual way or by electrical stimulation, and this was also the case in a patient with tabes dorsalis. This means that the circulation can be regulated through sensory impulses from the working muscles and that the impulses are certainly not conducted along those paths in the spinal medulla destroyed in tabes. The experiments also demonstrate that metabolic products, anoxaemia and dilatation of the vessels in the working muscles do not necessarily influence the regulation.

Probably the rise in blood-pressure has a bearing upon the maintenance of the increased gaseous metabolism of the tissues. The increase of pressure in the root of the aorta during work will always be greater than the increased pressure loss through the big arteries. How this influences the pressure in the capillaries in the working parts can only be guessed at, since it depends on how the pressure fall varies in the arterioles and to what extent the increased capillary flow is due to the opening of new capillaries or to the extreme dilatation of capillaries already open. However, it is granted that an increased pressure in the arterial part of the capillaries will be able to maintain the flow at a given level with a smaller available volume than if the pressure was unchanged and the flow increased through capillary dilatation alone. Through such regulation it is possible to maintain an adequate blood supply in all parts of the organism.

The fall of the blood-pressure at the end of work occurs more rapidly than the rise at the beginning of work in spite of the invariably raised metabolism of the recently working muscles



and the considerable oxygen deficit. The cause of the fall occurring so rapidly is most probably the dilatation of the muscular vessels after work and the cessation of the muscular movements which during work help to pump the blood towards the heart.

The rapidly occurring fall in pulse-rate at the end of work is certainly of significance too. The change in pulse-rate may be produced by the disappearance of those factors which during work sustained the blood-pressure at a high value. The pressure would be expected to fall considerably below the resting level. One of the reasons why this does not happen is surely that the pulse-rate after the cessation of work is invariably maintained considerably above the resting level. This regulation most likely takes precedence over the pressor-sensitive zones. Such a mode of regulation explains why the very constant relation during work between pulse-rate and oxygen intake ceases with the end of work, since the oxygen intake decreases far more rapidly than the pulse-rate (*Lythgoe & Pereira 1925, Kagan & Kaplan 1930*), and also why a fall in pulse-rate to normal values occurs later when the rate of work has been high and therefore many more muscular vessels have been dilated.

## SUMMARY

The aortic pressure variations are recorded from dogs during work and in the resting dog subjected to the influence of drugs acting upon the circulation and to aortic insufficiency by means of a manometer with electrical transmission. In order to get a gauge for the metabolic rate in the working experiments, the oxygen intakes have been determined at the different working rates using a mask and Douglas bag.

The aortic pressure is determined during the transition from rest to work, the steady state at different working rates and the transition from work to rest.

*The systolic blood-pressure* rises about 5 seconds after the beginning of work. In the course of 3—7 minutes it reaches a level, which with constant working rate remains unchanged to the end of work and then after 0—3 seconds falls and reaches the resting level 3—14 seconds after the cessation of work. The increase in *diastolic pressure* is smaller, so that the pulse-pressure obtains higher values during work and decreases after cessation of work. *The mean pressure* remains constant or is insignificantly increased in the period after the beginning of work which precedes the rise in systolic and diastolic pressures.

The *pulse-rate* increases from the resting level about 0,8 seconds after the beginning of work, reaches a peak value in 10—25 seconds and settles with constant working rate at a constant level after about 3 minutes. At the end of work it falls suddenly with large deviations, but does not reach the resting level in the recording time (10—20 seconds). In the individual animal a linear correlation is found between "steady state" values of systolic and diastolic pressures and the corresponding oxygen intakes, while the connection between pulse-rate and oxygen intake is less well defined. The procentual increase in blood-pressure in 21 experiments on 5 dogs varies approximately linear with the oxygen intake per kg.

At the start of work the *shape of the pressure pulse curve* changes, the systolic rise grows steeper and higher, the pressure fall from the maximum to the incisura increases and the diastolic fall is greatly diminished. These alterations appear before any change in blood-pressure level can be detected. The changes in the steep systolic rise depend on the pulse-rate. The secondary waves increase in amplitude.

*Amylnitrite* produces a pronounced increase in pulse-rate and a slight rise in blood-pressure. The whole of the systolic pressure rise takes place in the minimum ejection phase and the gradient increases in this period. The pressure fall from the systolic pressure maximum to the closure of the semilunar valves is increased considerably so that the pressure fall in the following part of diastole diminishes. The secondary oscillations increase in amplitude. *Nitroglycerine* produces a fall in blood-pressure. Changes occur corresponding to those produced by amylnitrite. Under the influence of large doses of *histamine* and *acetylcholine* the relations are the same except that the rise in the minimum ejection phase decreases when the pressure falls remarkably, and that the secondary oscillations under these conditions decrease in frequency. In the preceding experiments *adrenaline* produces no changes in the shape of the pressure pulse curve under normal conditions. When the pressure has been reduced by the action of acetylcholine the pressure rise increases, and the gradient in the minimum ejection phase and the positive diastolic pressure wave are increased. *In experimentally produced aortic insufficiency* the amplitude increases and the pressure course in the rising part of the curve increases in height and gradient, while the falling part of the curve corresponds to the course under the influence of vasodilator drugs. The action of nitroglycerine in aortic insufficiency produces the same changes as when the valvular function is normal.

The causes of the pressure course during work are discussed in comparison with earlier investigations on the pressure course in anaesthetised dogs under different conditions and with the changes found in the present investigation. Later on the essential cause for the shape of the aortic pressure pulse curve and the regulation of the circulation during work are discussed.

## DANISH SUMMARY

Afhandlingens Formaal er at undersøge Muskelarbejdes Indflydelse paa de absolutte Tryksvingninger i Aortaroden hos Hunde og at belyse, hvorledes Kredsløbet indstiller sig paa de ændrede Krav. De absolutte Trykvariationer er maalt i Hvile, i Overgangsperioden til Arbejde, under Arbejde af forskellig Intensitet og i Overgangsperioden til Hvile. Desuden er optaget Trykkurver fra den hvilende Hund under Indflydelse af Stoffer med Kredsløbsvirkning og under Aortainsufficiens, hvor de indtrufne Ændringer i Trykforholdet skyldes kendte eller delvis kendte Ændringer i de kredsløbsregulerende Faktorer. Trykket er registreret fra Aortaroden, da Trykforløbet her i højere Grad end noget andet Sted i Arteriesystemet maa anses for at være Udtryk for det af Hjertet udførte Arbejde, idet den Modulation, som den primære Trykbølge er underkastet paa Grund af Bølge-reflektion og Dæmpning i det elastiske Karsystem, anses for at være mindst her.

I Kapitel 1 omtales tidligere Undersøgelser over Blodtrykkets og Pulsens Forhold under Arbejde hos Dyr og Mennesker. I Dyreforsøg, hvor Arbejdet bestaar i Løb paa en Trædemølle, stiger Middeltrykket og Pulsfrekvensen efter Arbejdets Begyndelse og falder efter dets Ophør. Under elektrisk induceret Arbejde udført af narkotiserede Dyr, hvor medulla spinalis er overskaaret ved vb. thoracalis 12, finder man, at Trykket falder, saa snart Arbejdet sætter ind, hvorefter det holder sig lavt eller stiger til Hvileniveauet og undertiden lidt over dette i »Arbejdets« Forløb. Hos trænede Forsøgspersoner stiger Afklemningstrykket i de første Arbejdsminutter til en Værdi, der holdes konstant, saa længe Arbejdsydelsen er uforandret. Afklemningstrykket stiger lineært med Arbejdsintensiteten uanset, om Arbejdet er elektrisk induceret eller udføres paa sædvanlig Maade.

Det oscillometrisk bestemte Gennemstrømningstryk stiger slet ikke eller kun ubetydeligt under Arbejde, og det auscultatorisk bestemte diastoliske Blodtryk stiger kun lidt eller falder. Pulsfrekvensen forholder sig som Afklemningstrykket. Ved Arbejdets Ophør falder Afklemningstryk og Pulsfrekvens og naar Hvileværdien efter faa Minutter og for Blodtrykkets Vedkommende uafhængigt af det forudgaaende Arbejdes Størrelse. Pulsfrekvensen naar Hvileværdien senest efter de største Arbejdsintensiteter.

I Kapitel 2 redegøres for tidligere Undersøgelser vedrørende Aortatrykpulskurvens Form. Først efter at *Frank* konstruerede Manometre med tilstrækkelig Følsomhed, kunde der optages Trykpulskurver, der svarede til Trykforløbet. De første Maalinger paa unarkotiserede Dyr foretoges af *Gregg et al.* 1937. Forløbet bestaar af et systolisk Afsnit, som svarer til Trykforløbet i venstre Ventrikel i Uddrivningsperioden, og et diastolisk Afsnit, som repræsenterer Tidsrummet, i hvilket Semilunarklapperne er lukkede. En nøje Afgrænsning af Diastolen mod den næste Systoles Begyndelse lader sig ikke gennemføre uden samtidig Optagelse af Ventrikeltrykkurver, da Anspændingstidens Begyndelse ikke markeres i Aortatrykkurven. Den første Del af Systolen, Anspændingstiden, er derfor i dette Arbejde regnet med til Diastolen ved Bestemmelserne af de enkelte Perioders Varighed. Imellem Tidspunktet for Systolens Ophør og Semilunarklapperne Lukning falder Aortatrykket brat. Denne Periode benævnes Incisuren eller Protodiastolen. Incisuren er tidligere regnet til Systolen men bør rettelig tillægges Diastolen. Uddrivningsperioden inddeles efter *Wiggers* i 3 Afsnit, der ofte tydeligt kan adskilles, og som benævnes minimum ejection phase, maximum ejection phase og reduced ejection phase. Paa Trykkurvens Hovedforløb findes For-, Begyndelses- og Efter-svingninger paa de Steder, hvor der sker pludselige Ændringer i Hovedforløbets Trykgradient. De sekundære Svingninger opfattes som stærkt dæmpede Egensvingninger i det blodfyldte Hjerte-Karsystem. Paa Hjerte-Lungepræparater er det vist, at Trykket i venstre Ventrikel stiger hurtigere, naar Aortatrykket eller den diastoliske Fyldning forøges. Det samme gælder Aortatrykket hos narkotiserede Dyr under de samme Betingelser. I

den faldende Del af Blodtryksskurven tiltager Trykfaldet, før Klaplukningen finder Sted under Paavirkning med kardilaterende Stoffer, Shock og Blodtab. Ved experimentel Aorta-insufficiens hos narkotiserede Dyr er Amplituden forøget, og den første Del af Tryksskurven svarer til Forholdene hos Hunde med bevaret Klapfunktion, hvor den diastoliske Fyldning er forøget, mens den resterende Del svarer til Forholdene, hvor den cirkulerende Blodmængde er nedsat.

I Kapitel 3 omtales den benyttede Forsøgsteknik. I Træningsperioden opøves Forsøgsdyrene i Løb paa en Trædemølle med Hastigheder mellem 2 og 20 km/Time og en Baandhældning paa 4—16°. Løbehastigheden bestemmes ved at maale Tiden for 5—10 Omdrejninger. Dyrene vænnes ligeledes til at omgaas de Personer, der er til Stede ved Forsøgene og gøres fortrolige med Stofskiftebestemmelser og de Berøringer, der er nødvendige under Blodtryksmaalingerne. Træningsperioden varer for det enkelte Dyr 1—2 Maaneder. Iltoptagelsen maales under Løb af forskellig Hastighed og Baandhældning ved, at Dyrene, som bærer en specielt konstrueret Maske, eksspirerer i en Douglas-sæk. Af den i en vis Tid opsamlede Mængde Eksspirationsluft og af In- og Eksspirationsluftens Indhold af Ilt og Kuldioksyd beregnes Iltoptagelsen. Registrering af de absolutte Trykssvingninger i Aortaroden sker ved Hjælp af et Kondensatormanometer, som kobles til et Rør, som forud er indlagt i a. carotis, saa den ene Ende ligger i Aortaroden, og den anden munder ud paa Dyrets Hals. Trykvariationerne frembringer Kapacitetsvariationer, som omsættes til Spændingsvariationer, der optegnes paa fotografisk Papir ved Hjælp af en Oscillograf. For at hindre Koagulation i Rørsystemet under Maalingerne er Dyrene hepariniserede. Det benyttede Registreringsaggregat registrerer Trykvariationer mellem 0 og 250 Hz., og Forholdet mellem registrerede Udslag og statistiske Trykværdier er lineært mellem 0 og 300 mm Hg. Følsomheden, der kan varieres, er i Forsøgene altid saa stor, at der faas et Udslag paa 10 mm ved en Tryktilvækst paa 30—40 mm Hg.

I Kapitel 4 redegøres for Ændringerne i Pulsfrekvens, systolisk og diastolisk Blodtryk og Amplitude under Arbejde. Pulsfrekvensen stiger 0—3 (0,8) Sekunder efter Starten: Stigningen

er først stejl senere aftagende, til der naas et Maximum, hvis Højde er uafhængig af den opnaaede konstante Løbehastighed. Frekvensforøgelsen opnaaes til at begynde med næsten udelukkende gennem en Forkortning af Diastolen. Ved de højere Pulsfrekvensværdier er ogsaa Systolens Varighed nedsat. Det systoliske Blodtryk stiger efter 1—10 Sekunder over Hvileværdien og altid efter, at Pulsfrekvensen er steget. Det diastoliske Blodtryk forholder sig i Hovedsagen som det systoliske, men Stigningsgraden er mindre, hvorfor Trykamplituden tiltager. Middeltrykket holder sig uforandret, til Pulsfrekvensen stiger, hvorefter det ogsaa forøges. Det opnaaede Pulsfrekvensmaximum afløses efter 20—30 Sekunder af et Fald til en Værdi, der er 30—60 Perioder lavere, og som naas indenfor 2—3 Minutter. Det systoliske Blodtryk naar i 5 Forsøg et Maximum, hvorefter det falder indtil 30 mm til et Niveau, der naas indenfor 3 Minutter. I de øvrige Forsøg fortsætter den første Stigning men med aftagende Stejlhed og naar efter 3—7 Minutter et Niveau 10—60 mm over den Værdi, der naas i det første halve Minut. Det diastoliske Blodtryk forholder sig som det systoliske, saa den i Overgangsperioden fremkomne Amplitudetilvækst bevares uændret. Det opnaaede Pulsfrekvens- og Blodtryksniveau opret holdes i de fleste Forsøg under Resten af Arbejdet, hvis Arbejdsintensiteten ikke ændres. Niveaues Højde afhænger af Arbejdsintensiteten ligegyldigt, om det især er et Fremdrifts- eller Løftearbejde. *Der findes et lineært Forhold mellem systolisk og diastolisk Blodtryk og Iltoptagelsen per Minut og en noget mindre konstant Korrelation mellem Pulsfrekvens og Iltoptagelse. Forholdet mellem de i samtlige Forsøg fundne relative Uærdier for systolisk og diastolisk Blodtryk og Iltoptagelsen er praktisk taget lineært i hele det undersøgte Arbejdsomraade.* Stigningsgraden i Blodtryk svarer til de Værdier, der i Menneskeforsøg gælder for Afklemningstrykket ved tilsvarende Arbejdsydelser, mens Resultaterne slet ikke svarer til, hvad der er fundet at gælde for det auscultatorisk bestemte diastoliske Blodtryk og Gennemstrømningstrykket. I Overgangsperioden fra Arbejde til Hvile falder Pulsfrekvensen som Regel før Blodtrykket og til at begynde med hurtigt senere langsomt med ret store Udsving omkring en Middelværdi og ligger efter 10—20

Sekunder ret betydeligt over Hvileværdien. Det systoliske Blodtryk naar Hvileværdien eller en lidt højere Værdi paa 3—14 Sekunder. Det diastoliske Blodtryk følger i Hovedsagen det systoliske, men Amplituden aftager samtidig med, at Pulsfrekvensen nedsættes. Det systoliske og diastoliske Blodtryk falder hurtigere ved Arbejdets Ophør, end det steg ved Begyndelsen af Arbejdet. Mens Pulsfrekvensen synes at falde hurtigst efter de mindste Arbejdsydelser, kan der ikke paavises nogen Korrelation mellem det forudgaaende Arbejdes Intensitet og Blodtryksfaldet.

I Kapitel 5 redegøres for Trykpulskurvens Forløb i Hvile. Pulsfrekvensen er 77—156 (gennemsnitlig 120), det systoliske Blodtryk er 113—193 (147) og det diastoliske Blodtryk 87—156 mm (102 mm). I minimum ejection phase stiger Trykket stejlt, i maximum ejection phase langsommere og i reduced ejection phase aftager Trykket. I den paafølgende Protodjastole falder Trykket stejlt, til Semilunarklapperne lukkes. Paa den følgende Del af den diastoliske Trykkurve findes en positiv diastolisk Trykbølge undtagen, hvor Trykniveauet er meget højt.

I Kapitel 6 omtales Trykpulsens Form under Arbejde. Trykstigningen i minimum ejection phase tiltager, og Stigningsgraden forøges. Ofte naas Trykmaximum i denne Fase, saa maximum ejection phase forsvinder. Systolens Ophør markeres ikke. Trykfaldet fra det systoliske Trykmaximum til Tidspunktet for Semilunarklappernes Lukning forøges betydeligt saaledes, at Trykfaldet i Diastolen efter Klaplukningen formindskes og kan blive minimalt, hvis Amplituden ikke er forøget væsentligt. Den positive diastoliske Trykbølge forsvinder eller aflades, og de sekundære Svingninger tiltager i Amplitude.

I Kapitel 7 redegøres for Kurveformen under Paavirkning med Stoffer med Kredsløbsvirkning. Amylnitritpaavirkning bevirker en udtalt Pulsfrekvensforøgelse og en let Blodtrykstigning. Hele den systoliske Trykstigning sker i minimum ejection phase, og Hældningen tiltager i denne Periode. Trykfaldet fra det systoliske Trykmaximum til Tidspunktet for Semilunarklappernes Lukning forøges betydeligt, saa Trykfaldet i den følgende Del af Diastolen mindskes. De sekundære Svingninger tiltager i Amplitude. Nitroglycerin fremkalder et Blodtryksfald.



Før Indgiften var Blodtrykket højt og den positive diastoliske Bølge ikke tydelig. Under Paavirkningen indtraf der Forandringer i Forløbet svarende til Resultaterne ved Amylnitritpaavirkning med Undtagelse af, at den positive diastoliske Bølge blev mere udtalt, som Trykket faldt. Under Paavirkning med store Doser Histamin og Acetylcholin er Forholdene de samme som ved Amylnitritpaavirkning med Undtagelse af, at Stigningen i minimum ejection phase aftager, naar Trykket falder meget, og at de sekundære Svingninger under disse Forhold aftager i Frekvens. Paavirkning med Adrenalin bevirker i de foreliggende Undersøgelser ingen Ændringer paa det unarkotiserede Dyr. Hvor Trykket i Forvejen er lavt paa Grund af Acetylcholinpaavirkning, forøges Trykstigningen og Stigningsgraden i minimum ejection phase og den positive diastoliske Trykbølge forøges.

I Kapitel 8 omtales de Forandringer, der fremkommer i Tryk-pulsen ved eksperimentelt fremkaldt Aortainsufficiens. Forandringerne svarer til de, der er fundet tidligere under tilsvarende Betingelser hos narkotiserede Dyr. Amplituden tiltager, og Trykforløbet i den stigende Del af Trykkurven tiltager i Hældning og Højde, mens den faldende Del af Kurven svarer til Forløbet under Paavirkning med kardilaterende Stoffer, idet den største Del af Trykfaldet sker hurtigt, saa Faldet i den sidste Del af Diastolen bliver ringe. Nitroglycerinpaavirkning under Aortainsufficiens fremkalder de samme Forandringer, som naar Klapfunktionen er naturlig.

I Kapitel 9 diskuteres Aarsagen til Trykforløbet under Arbejde ud fra Sammenligning med tidligere Undersøgelser af Trykforløbet paa narkotiserede Dyr under forskellige Betingelser og de i dette Arbejde fundne Ændringer i Forløbet ved Paavirkning med Stoffer med Kredsløbsvirkning og Aortainsufficiens. Det antages, at Forandringerne skyldes, at venstre Ventrikel tømmer sig hurtigere end i Hvile og maaske med et forøget Slagvolumen samtidig med, at den perifere Modstand er nedsat. Senere i samme Kapitel diskuteres den egentlige Aarsag til Aortatrykpulskurvens Form, som tilskrives Interferens mellem en primær Bølge og en sekundær Bølge, som er Summen

af talrige reflekterede Bølger. Forskellen i Trykforløbet under forskellige Forsøgsbetingelser antages at bero paa Ændring i Amplitude, Forløbsform eller Faseforskydning paa Grund af ændret Hjertefunktion eller ændrede Bølgetransmission- eller Reflektionsmuligheder. Det understreges, at Pulsbølgen ikke er en rytmisk Bølgebevægelse, men en Række Enkeltfænomener, der skyldes Hjertets Arbejde, ligesom man ikke kan tale om en Forplantning af Trykbølgen hen over Karsystemet, idet Pulsbølgen paa et givet Sted er opbygget af talrige Trykkomponenter med forskellig Retning og Styrke, som er karakteristiske for Maalestedet, og at visse af disse Komponenter ikke findes paa Maalesteder med anden Lokalisation.

I Kapitel 10 omtales Kredsløbsregulationen under Arbejde. Den hurtigt indtrædende Stigning i Pulsfrekvens kan kun tænkes udløst ad nervøs Vej gennem en Nedsættelse af Vagustonus. De udløsende Impulser kommer sandsynligvis fra de arbejdende Muskler. Det er usandsynligt, at Frekvensforøgelsen udløses gennem en Impulsændring fra de pressosensible Zoner, da Frekvensen stiger uden et forudgaaende Blodtryksfald.

Blodtryksstigningen, der indtræder efter Stigningen i Pulsfrekvens, maa tages som Udtryk for, at der stadig tilføres Aortaroden mere Energi, end der forsvinder paa Grund af, at Minutvolumen forøges. Da Trykamplituden tiltager efter, at Trykstigningen er begyndt, maa det ligeledes antages, at den diastoliske Fyldning er tiltaget. Den forøgede venøse Fyldning maa tilskrives Dilatation af Muskelkarrene og Kontraktion af Karrene i Portagebetet og i ikke arbejdende Organer. At den samlede perifere Modstand er nedsat tidligt i Arbejdet synes at fremgaa af, at det diastoliske Trykforløb under Arbejde svarer til Forholdene ved Paavirkning med kardilaterende Midler og Trykforløbet ved Aortainsufficiens.

Det er muligt, at de pressosensible Zoner indgaar i den Reguleringsmekanisme, som bevirker, at Blodtrykket under Arbejde i steady state holdes paa et konstant Niveau afhængigt af Arbejdets Intensitet gennem en Ændring i de overordnede Centres Følsomhed for Impulserne paa Grund af andre samtidige Paa-virkninger. Det er vist, at kredsløbsregulerende Impulser under

Arbejde udgaar fra de arbejdende Muskler, og at Ophobning af Stofskifteprodukter, Iltmangel og Kardilatation i de arbejdende Omraader ikke behøver at influere paa Reguleringen.

Blodtryksstigningen har sandsynligvis Betydning for Opretholdelsen af Vævenes forøgede aerobe Stofskifte ved at bevirke, at den nødvendige Gennemblødning kan finde Sted med en mindre disponibel Blodmængde, end det vilde være Tilfældet, hvis Trykket holdt sig uforandret.

Blodtrykket falder hurtigere ved et Arbejdes Ophør end ved dets Begyndelse. Grunden er sandsynligvis den, at de Faktorer, der under Arbejdet opretholder det forhøjede Blodtryk, falder væk samtidig med, at Muskelkarrene er dilaterede. At Trykket ikke falder betydeligt under Hvileniveauet maa tilskrives Regulering over de pressosensible Zoner, hvilket forklarer, hvorfor Pulsfrekvensen naar sin Udgangsværdi senest efter de største Arbejder, hvor særlig mange Muskelkar har været dilaterede.

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*From the Department of Pharmacology,  
Karolinska Institutet, Stockholm, Sweden*

The Effect of Synthetic Amino Acids  
Essential for Growth on the Body Weight  
of Growing Rats, and the Synthesis  
of the Amino Acids Used

BY

K. A. J. WRETLIND



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## CHAPTER I

### Historical Survey

#### Survey of previous attempts to replace protein in the diet by amino acids

In the course of the 19th century it was found that protein as a constituent in the diet was essential for maintenance of life and growth (MAGENDIE 1816, VON LIEBIG 1843, PROUT 1834). LOEWI (1902) showed that it could be completely replaced in the diet by hydrolysis products of protein for the building-up of body-protein. In his experiments he used autodigested pancreas. These results were verified by ABDERHALDEN (1912, 1913). In the enzymatic hydrolysis of protein, free amino acids only are never obtained: there is always a certain admixture of peptides. It therefore seemed desirable to ascertain how far protein in the diet could be replaced by a hydrolysate completely free from peptides and protein. If protein is hydrolyzed with acid, a mixture containing free amino acids only will be obtained. With such acid-hydrolyzed protein HENDERSON and DEAN (1903) tried to obtain a nitrogen-equilibrium and a protein synthesis in dogs, but failed, probably owing to the destruction of tryptophane in the acid-hydrolysate. Similar attempts with acid-hydrolyzed protein were made by ABDERHALDEN and RONA (1904). Later, HOPKINS (1916) showed that growth can be maintained in rats by acid-hydrolyzed protein fortified with tryptophane.

In the early years of the present century, methods for the analysis and isolation of the amino acids present in protein were elaborated. It was then considered suitable to endeavour, on the basis of available analyses, to replace the protein in the diet by a mixture of free amino acids. Thus, ABDERHALDEN (1912) tried to replace the protein in the diet by a mixture of 16 amino acids. The experiments were made on dogs. The animals refused to eat much of the food. Their nitrogen and body-weight

equilibrium was said to be maintained for periods of six to eight days. Similar attempts to supply amino acids were made by HOPKINS (1916), OSBORNE and MENDEL (1916), MITCHELL (1916), ABDERHALDEN (1922), SUZUKI, MATSUYAMA, and HASHIMOTO (1926). All these attempts showed that it was not possible, with the amino acid mixtures then used, to obtain growth in the experimental animals. The chief reason for their failure was obviously because all the essential amino acids had not as yet been discovered and isolated (methionine was isolated in 1922 by MUELLER and threonine in 1935 by Mc COY, MEYER, and ROSE). The fact that ABDERHALDEN (1912) maintained body weight in a dog with his amino acid mixture was probably due to contamination of the amino acids used with methionine and threonine.

The possibility of completely replacing protein by a mixture of amino acids was first shown by ROSE's investigations. In his first attempt (ROSE 1931), the protein in the diet was replaced by a mixture of amino acids containing glycine, dl-alanine, dl-valine, l-leucine, dl-isoleucine, dl-norleucine, l-proline, l-hydroxyproline, dl-phenylalanine, l-glutamic acid, l-aspartic acid, dl-serine, l-tyrosine, l-cystine, l-histidine, l-arginine, l-methionine, l-lysine, and l-tryptophane. The proportions of the amino acids were approximately the same as in casein according to the then current analyses. The diet in ROSE's (*loc. cit.*) experiment consisted of the forementioned amino-acid mixture (24.1 %), glucosamine HCl (1 %), sodium bicarbonate (0.4 %), dextrin (22.5 %), saccharose (15 %), a salt mixture (4 %), agar (2 %), lard (26 %), and cod liver oil (5 %). Vitamins of the B-group were given in the form of 200 mg of yeast per day. On this diet the experimental animals, albino rats (68—96 g), lost in weight and ate less than usual. The conclusion drawn from these tests was that protein must contain at least one essential factor in addition to the then known amino acids.

Continued tests by ELLIS and ROSE (1931) showed that if the above-mentioned amino-acid diet used by ROSE was mixed with 5 % of gliadin or casein, it would tend to promote growth. According to those investigators, the result was due to the fact that gliadin and casein contained an unknown factor stimulating growth. In order to isolate this factor, WINDUS, CATHERWOOD,

and ROSE (1931) hydrolyzed casein, and the amino acids thus obtained were separated in accordance with known methods into five different groups, namely the sparingly soluble, the dibasic and the diamino acids, those soluble in alcohol and the other monoamino acids. It was then found that the hypothetical growth-promoting factor was contained in the monoamino acid fraction (the fraction soluble in butyl alcohol).

In the course of the next few years attempts were made to isolate this new factor (CALDWELL and ROSE 1934). A difficulty which nearly upset the whole investigation was that the fore-mentioned mixture of amino acids contained an unduly low concentration of isoleucine. Growth was therefore obtained only when the impure preparations of the growth factor also contained isoleucine. In this way the identification was greatly impeded. When this defect had been brought to light (WOMACK and ROSE, 1935) and when it had further been shown that fibrin was a better source of the growth factor, the two active principles were separated. One of them proved to be isoleucine. Shortly afterwards the growth factor was isolated in a pure form by MC COY, MEYER, and ROSE (1935) and was found to be one of the four isomers of  $\alpha$ -amino- $\beta$ -hydroxy-n-butyric acid (dl-threonine and dl-allothreonine). Owing to its structural affinity with d(—)-threose, it was termed d(—)-threonine. It was thus for the first time possible to make experiments where the protein in the diet was, practically speaking, completely replaced by a mixture of amino acids prepared in a pure form.

### Essential and non-essential amino acids

With the forementioned experiments of ROSE and co-workers, a good method had been provided for determining how far the different amino acids were indispensable for the organism. They composed a diet containing all the amino acids, with which they obtained growth in rats. If the amino acid of which the indispensableness for growth was to be investigated was excluded from the diet they obtained in certain cases no change in the growth, in others, a poorer growth or a decline in the body weight. If no change in the growth resulted from this exclusion, the amino acid in question was termed non-essential. If poorer growth



or a fall in weight ensued, the amino acid was regarded as essential.

With this technique it was found that the following amino acids were indispensable or essential for the growth of rats, namely phenylalanine (WOMACK and ROSE 1934, ROSE and WOMACK 1946), leucine and isoleucine (WOMACK and ROSE 1936), methionine (WOMACK, KEMMERER, and ROSE 1937), and valine (ROSE and EPPSTEIN 1939). It had been clearly shown by the previous investigations of OSBORNE and MENDEL (1912, 1914) that both lysine and tryptophane were essential for growth in rats. The tests were made with zein, which is devoid of tryptophane and lysine, and with gliadin, which contains no lysine. If these proteins were the only source of nitrogen included in the diet, no growth was obtained. Only after the addition of the fore-mentioned missing amino acids was an increase in weight observed.

ACKROYD and HOPKINS (1916) noted that if arginine and histidine were removed from acid-hydrolyzed casein, it was not possible with the remaining mixture to produce growth in rats, even when tryptophane was supplied. If, on the other hand, arginine or histidine were added, they obtained the desired growth. They therefore presumed that arginine and histidine could replace one another. ROSE and COX (1924, 1926) made the same experiment and verified that histidine promoted growth under such conditions, but did not find that arginine had any effect on the growth in default of histidine. Thus, histidine but not arginine could be designated as essential. Further evidence in support of the essential character of histidine was published by HARROW and SHERWIN (1926). Arginine was afterwards found to be essential only for optimal growth in the rat (ROSE 1938, BORMAN, WOOD, BLACK, ANDERSON, OESTERLING, WOMACK, and ROSE 1946).

Amino acids which were found to be lacking in importance for growth (i. e. non-essential) were glycine and serine (Mc COY and ROSE 1937), aspartic acid (ROSE and FIERKE 1942), norleucine (WOMACK and ROSE 1936), cystine (ROSE and RICE 1939) and hydroxyproline (WOMACK and ROSE 1947). Tyrosine was found to be non-essential (WOMACK and ROSE 1934), but later investigations (WOMACK and ROSE 1946) showed that if phenylalanine were

supplied in suboptimal amounts, the growth could be stimulated by the addition of tyrosine.

Cystine (according to WOMACK, KEMMERER, and ROSE 1937) is of no importance for growth. It appeared, however, from later investigations by WOMACK and ROSE (1941) that, if methionine was included in the diet in suboptimal amounts, an increase in the growth could be obtained on the addition of cystine. Proline likewise tends to increase the growth if arginine is lacking in the diet (WOMACK and ROSE 1947). ROSE, OESTERLING, and WOMACK (1948) showed that, if glutamic acid was excluded from a mixture of 19 amino acids, the rate of growth would be decreased merely in a slight degree.

On the basis of this finding, ROSE, OESTERLING, and WOMACK (1948) then drew up the following table of amino acids with respect to their importance for optimal growth of the rat. The classification is the same as that earlier proposed by ROSE (1938).

TABLE I. Grouping of amino acids in essential and non-essential according to ROSE (1938) and ROSE, OESTERLING, and WOMACK (1948).

Essential amino acids	Non-essential amino acids
Arginine <sup>1</sup>	Alanino
Histidine	Aspartic acid
Isoleucine	Citrulline
Leucine	Cystine <sup>2</sup>
Lysine	Glutamic acid <sup>3</sup>
Methionine	Glycine
Phenylalanine	Hydroxyproline
Threonine	Norleucine
Tryptophane	Proline <sup>4</sup>
Valine	Serine
	Tyrosine <sup>5</sup>

<sup>1</sup> Arginine may be synthesized by the rat itself, but not rapidly enough to bring about optimal growth.

<sup>2</sup> Cystine may stimulate the growth if the concentration of methionine is suboptimal.

<sup>3</sup> Glutamic acid can accelerate the growth if only the 10 essential amino acids occur in the diet.

<sup>4</sup> Proline will accelerate the growth in default of arginine in the diet.

<sup>5</sup> Tyrosine stimulates the growth if the amounts of phenylalanine are suboptimal.

As regards these essential amino acids, it has been shown that both the l- and the d-forms of tryptophane (BERG and POTGIETER 1932, BERG 1934), methionine (JACKSON and BLOCK 1933), histidine (COX and BERG 1934, CONRAD and BERG 1937), and phenylalanine (ROSE and WOMACK 1946) can produce growth in rats with diets containing all the other ingredients in addition to these amino acids. The d-form of histidine has a somewhat poorer effect than the l-form (COX and BERG, *loc. cit.*). ALBANESE, IRBY, and FRANKSTON (1945) have shown that both d- and l-arginine are utilized. It is only the naturally occurring isomer of lysine that has growth-promoting properties (Mc GINTY, LEWIS, and MARVEL 1924, BERG 1936). According to ROSE (1938), only the l-form of valine, leucine and isoleucine has the capacity to produce growth. Investigations by RATNER, RITTENBERG and SCHOENHEIMER (1940) with deuterium and N<sub>15</sub> introduced into d-leucine show, however, that this isomer can partly be converted in adult rats into l-leucine. Of the two isomers of dl-threonine only d(—)-threonine tends to promote growth (WEST and CARTER 1937).

In a review of the subject, ROSE (1937) indicated the minimum content of the essential amino acids in the diet for optimal growth of rats. In these tests the non-essential amino acids were also present. The said minimum concentrations of the essential amino acids in the diet are shown in the following table II. In this table, the concentrations refer to the naturally occurring forms.

TABLE II. Minimum concentration of ROSE's essential amino acids in the food required for optimal growth in rats.

Amino acid	%	Amino acid	%
Lysine . . . . .	1	Isoleucine . . . . .	0.5
Tryptophane . . . . .	0.2	Threonine . . . . .	0.6
Histidine . . . . .	0.4	Methionine . . . . .	0.6
Phenylalanine . . . . .	0.7	Valine . . . . .	0.7
Leucine . . . . .	0.9	Arginine . . . . .	0.2

**Attempts to obtain growth in rats on a diet where the protein had been replaced solely by Rose's essential amino acids.**

In connection with the classification of the different amino acids into essential and non-essential acids, one or two of them, as previously described, were removed from the diet and the resulting effect on the growth noted. If it was found that only 10 of them were essential, the obvious procedure was to see how far it was possible, with these 10 amino acids as the sole source of nitrogen in the food, to obtain growth. Such an attempt was made by MEYER and ROSE (not published, but communicated by ROSE in 1938) with a diet containing 11.2 % of a mixture of the 10 essential amino acids. The growth is stated to have been the same as when 20 amino acids were included in the diet. No details of the tests have, however, been reported.

In 1943 ALBANESE and IRBY published an investigation into the growth of rats where the protein in the diet had been replaced by a mixture of Rose's essential amino acids with the addition of cystine. The amino acid mixture used had the following composition:—

d-arginine HCl .....	12.7 g
d-histidine HCl .....	6.8 g
l-leucine-isoleucine mixture .....	14.0 g
l-leucine .....	12.0 g
l-lysine HCl .....	19.2 g
dl-methionine .....	7.0 g
dl-phenylalanine .....	15.6 g
dl-threonine .....	12.0 g
l-tryptophane .....	4.5 g
dl-valine .....	32.0 g
l-cystine .....	2.5 g

This mixture formed 14.7 % of the diet and was then tested on white rats with an initial weight of 63—79 grams. In this test it was found that in 12 days the rats had lost on an average 16.5 grams in weight. In order to rule out the possibility that any of the amino acids occurred in an unduly low concentration, the content of the amino acid mixture in the diet was increased to double (29.4 %). Out of six rats (with an initial weight of 54—60 grams) put on this diet, three died within 5 days, whereas the other three survived for 3 weeks, whereupon they were killed. They had lost more in weight than those on 14.7 % of the

diet. ALBANESE and IRBY (*loc. cit.*) attribute the lethal effect of the amino acids in this case to toxicity of the not naturally occurring isomers of the *racemic* forms which cannot be utilized by the animal: it may be seen from the above list that four of the amino acids were supplied in the dl-form.

KINSEY and GRANT (1944) published a similar investigation into the possibility of feeding rats on a diet where the essential amino acids were the only source of nitrogen. The mixture used had the following composition:—

l-arginine HCl .....	0.242 g
l-histidine HCl .....	0.494 g
dl-isoleucine .....	1.000 g
l-leucine .....	0.900 g
l-lysine HCl .....	1.250 g
dl-methionine .....	0.600 g
dl-phenylalanine .....	0.700 g
dl-threonine .....	1.200 g
dl-tryptophane .....	0.200 g
dl-valine .....	1.400 g

These amino acids were then mixed with a nitrogen-free diet, to which vitamins in a pure form were added. The investigated concentrations of amino acids in the diet were 8 and 16 %. It was then found that the rats (initial weight 35—37 g) in 21 days gained on an average 8 grams in weight on the 8 % diet. When the concentration was raised to 16 %, two of the rats gained in weight 26 and 19 grams, respectively, in 21 days. It seems to have been clearly shown by this experiment that growth in rats can be obtained even on a diet with Rose's essential amino acids as the sole source of nitrogen. Six of the amino acids supplied were synthetic racemic forms.

MARTIN (1944) fed rats with an initial weight averaging 52.0 g on a diet where the protein had been completely replaced by a mixture of the ten essential amino acids. His mixture had the following composition:—

l(+)-lysine HCl .....	50.5 g
dl-valine .....	45.0 g
l-leucine .....	36.0 g
dl-isoleucine .....	40.5 g
dl-threonine .....	23.0 g
l-tryptophane .....	9.0 g

l-histidine .....	15.75 g
dl-phenylalanine .....	33.75 g
dl-methionine .....	31.50 g
l(+)-arginine .....	13.50 g

The concentration in the diet was 16 %, and on this diet growth in rats was obtained. The tests proceeded for 7 weeks, and the gain in weight (5 rats) during this period averaged 48.9 grams.

In 1946 BORMAN, WOOD, BLACK, ANDERSON, OESTERLING, WOMACK, and ROSE showed that growth in rats can be obtained with a mixture of only the 9 essential amino acids, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine. With the exception of tryptophane and histidine, which were derived from protein, the amino acids were synthetic products. In the course of 28 days the gain in weight averaged  $28.9 \pm 0.41$  grams. When 0.24 % of l(+)-arginine was added to the diet, the gain in weight in 28 days increased to  $36.0 \pm 0.55$  grams. This shows both that rats can grow on a diet containing only the 9 essential amino acids, and that arginine is necessary if optimal growth is to be obtained.

ROSE, OESTERLING, and WOMACK (1948) report an investigation into the growth of rats on a diet containing only Rose's ten essential amino acids. Five of the 10 amino acids were supplied in the racemic form. As a basis of comparison they also used a mixture in which 19 amino acids were included. It was found in this comparative test that on the diet containing only the 10 amino acids the rats gained 70—75 per cent in weight of the result obtained on the diet with the 19 amino acids. On further analysis it was shown that if glutamic acid was added to the diet with the ten amino acids, the gain in weight was considerably greater than without this addition.

From the forementioned experiments it is quite evident that growth in the rat can be maintained with a diet in which the protein is replaced by a mixture of nine amino acids essential for growth. In all these experiments some of the amino acids were prepared from proteins and the others synthetically prepared. It seems necessary to add at least arginine and glutamic acid in order to obtain optimal growth.

## CHAPTER II

## The author's classification of the amino acids

Amino acids essential for growth. Amino acids essential for optimal growth. Non-essential amino acids

From the above report on the principal earlier experiments on the growth of rats on a diet in which the protein had been replaced by mixtures of pure amino acids, it is seen that the effect of the different acids on the growth shows great variations. In respect of this effect, the amino acids may be ranged in the following three groups:—

1. *Amino acids essential for growth.* Amino acids absolutely necessary for growth; i. e. if any of them is lacking in the diet, a loss of weight will result.

2. *Amino acids essential for optimal growth.* Amino acids required for optimal growth only. If they are lacking in the diet a decreased gain in weight will result.

3. *Non-essential amino acids.* Amino acids which have no effect whatever on the growth when amino acids from the two other groups are present in sufficient concentrations. Certain amino acids in this group, however, can produce better growth if some of the amino acids from the first two groups occur in suboptimal concentrations.

TABLE III. Grouping of amino acids into essential for growth, essential for optimal growth and non-essential, according to the author.

Amino acids essential for growth	Amino acids essential for optimal growth	Non-essential amino acids
Histidine Isoleucine Leucine Lysine Methionine Phenylalanine Threonine Tryptophane Valine	Arginine Glutamic acid	Alanine Aspartic acid Citrulline Cystine Glycine Hydroxyproline Norleucine Proline Serine Tyrosine

Thus, according to this classification, it should be possible with a mixture consisting only of the amino acids in the 1st and 2nd group to obtain optimal growth. In this event, addition of the amino acids in the 3rd group would not bring about any improvement in the growth.

The author's classification of the amino acids is thus that shown by Table III. It should be noted that the grouping applies only to the growth of the *rat*.

## CHAPTER III

### Outline of the problem

From the forementioned experiments it is evident that if the protein in the diet is completely replaced by the 10 essential amino acids specified by ROSE (*loc. cit.*) or merely by the 9 amino acids essential for growth, it will be possible to produce growth in rats. The mixtures used in all these previous experiments contained both amino acids isolated from protein and those synthetically produced. It should, however, be noted that, in using amino acids isolated from protein, there is always the risk that they may be contaminated with other amino acids. For an exact study of the amino acids from a dietary point of view, it is of vital importance to avoid such contamination. One way in which this object could be attained would be to confine oneself to the use of synthetically produced amino acids. Contamination with other amino acids could thus be prevented: moreover it would then presumably be easier in the future to obtain the amounts of pure amino acids required for such experiments.

As previously mentioned, certain isomers of the amino acids cannot be utilized by the body for growth. We are thus faced with the question how far these isomers are harmless or not. Attention has already been drawn to the supposition of ALBANESE and IRBY (*loc. cit.*) that the racemic forms were toxic. This important question—whether the racemic forms are really toxic or such harmless substances as to permit growth—can be decided only by feeding tests with synthetic amino acids as the sole



source of nitrogen.—In fact it has been directly shown that some racemic amino acids are more toxic than the naturally occurring isomers. Thus HOWE, UNNA, RICHARDS and SEELER (1946) showed that dogs tolerated intravenous injections of the dl-methionine less well than the l-methionine. ARTOM, FISHMAN and MOREHEAD (1945) found that the dl-serine produced fatal injury in rats characterized by necrosis of the kidney tubules. Under the same experimental conditions the administration of l-serine was not accompanied by such lesions.

In a preliminary communication, the author (WRETLIND, 1948) has for the first time shown that it is possible to obtain growth in rats on a diet in which the protein has been replaced by the amino acids essential for growth plus dl-arginine, all of them synthetically produced. In these experiments an amino acid mixture of the following composition was used:—

dl-arginine HCl	.....	2.6 g ( 7.7 %) <sup>1</sup>
dl-histidine HCl	.....	1.2 g ( 3.5 %) <sup>1</sup>
dl-isoleucine	.....	4.0 g (14.4 %)
dl-leucine	.....	4.0 g (14.4 %)
dl-lysine HCl	.....	2.8 g ( 8.1 %) <sup>1</sup>
dl-methionine	.....	2.0 g ( 7.2 %)
dl-phenylalanine	.....	1.6 g ( 5.8 %)
dl-threonine	.....	3.2 g (11.5 %)
dl-tryptophane	.....	0.8 g ( 2.9 %)
dl-valine	.....	5.6 g (20.1 %)

<sup>1</sup> Estimated as a free base.

As laboratory animals the author used white rats with an initial weight of 44.5—50.4 grams, the experimental period being 10 days. With an amino acid concentration of 10 and 20 % a growth of  $3.3 \pm 1.4$  and  $8.9 \pm 1.5$  grams in the first 10 days was obtained. If, on the other hand, the amino acid concentration in the food amounted to 30 or 40 %, the result was a loss in weight of  $1.1 \pm 2.5$  grams and  $6.3 \pm 2.7$  grams, respectively, in the first 10 days.

From these tests it seems to be evident that with 10 synthetic amino acids only it is possible to obtain growth in rats. It seems, moreover, as though the synthetic amino acids in *higher concentration* were toxic.

This investigation has been extended with the object of making a closer study of the changes in weight and the intake of food of rats on a diet where the protein had been replaced by different concentrations of the amino acids essential for growth, all of which had been synthesized according to the methods described. They were supplied in their racemic forms. The tests, which will be reported in detail below, showed that growth can be obtained on a diet containing merely these 9 synthetic amino acids. It should be noted, however, that dl-arginine was included in the diet mixtures in some of the tests in order to obtain as marked differences as possible in the rate of growth: it has previously been mentioned that BORMAN, WOOD, BLACK, ANDERSON, OESTERLING, WOMACK and ROSE (*loc. cit.*) had shown that arginine is essential for optimal growth. That glutamic acid, which is likewise of importance for optimal growth, was not administered is due to the fact that the work by ROSE, OESTERLING, and WOMACK (1948) in which this was pointed out was not published until after the termination of the tests. This, however, does not essentially affect the present author's attitude to the problem, nor his results.

The extended investigation is intended to bear out the evidence previously brought forward by the author, i. e. that rats can grow on synthetic amino acids as the sole substitute for protein in the diet. It is also intended to reply on broad lines to the following questions:—

1. How does the body-weight change with a varying content of the several amino acids essential for growth, from a concentration of 0 % until their toxic effect is observed?
  - A. How large is the loss in weight when any of the amino acid essential for growth is lacking?
  - B. What is the minimum concentration of each individual amino acid that is required in order to keep the body-weight unchanged?
  - C. What is the concentration for maximal growth?
  - D. At what concentration has the amino acid in question an indubitably toxic (stagnation of growth, loss in weight) or lethal effect?

2. How does the intake of food change with varying concentrations of the different amino acids essential for growth?

3. How great is the growth in rats on a diet of the 10 synthetic amino acids, as compared with ordinary diet and casein diet?

4. What is the growth with a diet, in which the protein is replaced by only the nine synthetic amino acids essential for growth?

## CHAPTER IV

### Synthesis of the amino acids used in the tests

All the amino acids used in the investigation reported here were synthesized by the author in accordance with the methods indicated below. — The risk of contamination with other essential amino acids seems to be practically non-existent in these syntheses. In the production of synthetic amino acids, however, it should be observed that in certain methods for the synthesis of dl-leucine, we are liable to obtain a product containing dl-isoleucine as an impurity. HEGSTAD and WARDWELL (1944), having examined 7 samples of dl-leucine, found only two that were quite free from dl-isoleucine. The others contained between 0.5 and 20 % of isoleucine. — This occurs especially if the synthesis is made from isocaproic acid, which is converted to dl- $\alpha$ -bromoisocaproic acid. By amination of this  $\alpha$ -bromo acid dl-leucine is produced. Isocaproic acid, depending on the process of production, may be contaminated up to 30 % by  $\beta$ -methylvaleric acid, which signifies that, via dl- $\alpha$ -bromo- $\beta$ -methylvaleric acid, dl-isoleucine is obtained. A similar risk exists in the synthesis of dl-leucine from isoamyl alcohol via isocaproic nitrile and isocaproic acid etc. In endeavouring to do so, the author found that the isocaproic acid thus obtained had been contaminated to 20—30 % by  $\beta$ -methylvaleric acid. In order to obviate any admixture of dl-isoleucine, the dl-leucine was synthesized from isobutyl bromide via a malonic acid synthesis (see p. 30). In a similar way dl-isoleucine was produced, though from sec. butyl bromide (see p. 28). Each of these butyl bromides can be obtained in a pure form. For these reasons, it

may be taken for granted that the dl-leucine and the dl-isoleucine used here were pure.

The syntheses have been made mainly according to previously described methods. Some new reactions and methods have, however, been introduced.

A special technique for the production of dl-arginine monohydrochloride — broadly speaking, similar to that proposed by Cox (1928) for the production of l-arginine monohydrochloride — has been elaborated. The method is based on the fact that, by the addition of aniline to a hydrochloric alcoholic solution of dl-arginine, the excess of hydrochloric acid is converted into aniline hydrochloride, which is soluble in alcohol, whereas the non-soluble dl-arginine monohydrochloride is precipitated. — dl-Arginine hydrochloride has previously been prepared by heating l(+)-arginine hydrochloride (FELIX and DIRR, 1928).

For the preparation of dl-histidine according to PYMAN (1911) 4-hydroxymethylglyoxaline was produced from diaminoacetone and potassium thiocyanate. The first stage in this synthesis was the production and isolation by alcohol extraction of 2-thiol-4-aminomethylglyoxaline, which was then oxidated with  $\text{HNO}_3$  to 4-hydroxymethylglyoxaline. With this procedure, however, poor yields were obtained. Moreover the method was troublesome, as the extraction with alcohol turned out badly, owing to the poor solubility of 2-thiol-4-aminomethylglyoxaline in alcohol. — It was found that the procedure could be modified by producing the 4-hydroxymethylglyoxaline direct from the diaminoacetone without previous isolation of the 2-thiol-4-aminomethylglyoxaline. Potassium thiocyanate and diaminoacetone were allowed to react to the 2-thiol-4-aminomethylglyoxaline, which, without isolation, was oxidized with nitric acid to 4-hydroxymethylglyoxaline, the latter being isolated as picrate. After extraction of the picric acid, 4-hydroxymethylglyoxaline HCl was obtained.

The synthesis of dl-histidine has also been performed according to ALBERTSON and ARCHER (1945). In their method (page 26) they use ethyl acetaminomalonate. This substance was produced mainly in accordance with SNYDER and SMITH's method (1944), but with a modification introduced by the author to make the method more convenient. According to SNYDER and SMITH (*loc. cit.*) ethyl malonate was treated with sodium nitrite

in glacial acetic acid, after which the ethyl isonitrosomalonnate was extracted with ether. The ether was evaporated, and the ethyl isonitrosomalonnate transferred to alcohol and hydrogenated in the presence of Pd-charcoal catalyst. When the Pd-charcoal had been filtered off, acetic anhydride was added, and after some time the ethyl acetaminomalonnate crystallized. As this substance, relatively speaking, is readily soluble in alcohol, the method was modified so that the acetylation was effected in ether, where the ethyl acetaminomalonnate is comparatively soluble in heat, but sparingly soluble in cold.

For the production of the free base dl-histidine, the author adapted the above-mentioned aniline method for removal of the hydrochloride from the dl-histidine dihydrochloride obtained in accordance with known methods. This method of removing the hydrochloride was much simpler than those previously adopted, which are based on the removal of the hydrochloride with silver oxide (PYMAN 1911 b, 1916, ABDERHALDEN and WEIL 1912).

The method also seemed to be more convenient than that proposed by ALBERTSON and ARCHER (*loc. cit.*). According to their method dl-histidine is produced by hydrolysis and decarboxylation of ethyl 2-acetamino-2-carbethoxy-3-glyoxalinepropionate with sulfuric acid. After the removal of the sulfuric acid as barium sulfate, dl-histidine is precipitated with alcohol.

The synthesis of dl-leucine has previously been described in essentials by FISHER and SCHMITZ (1906), but, as indicated above, the technique was modified by the author, so that it—broadly speaking—follows MARVEL's (1941 a) synthesis of dl-isoleucine, though with the difference that sec. butyl bromide has been replaced by isobutyl bromide. This method seemed to be simpler than the original technique, and the yield was satisfactory.

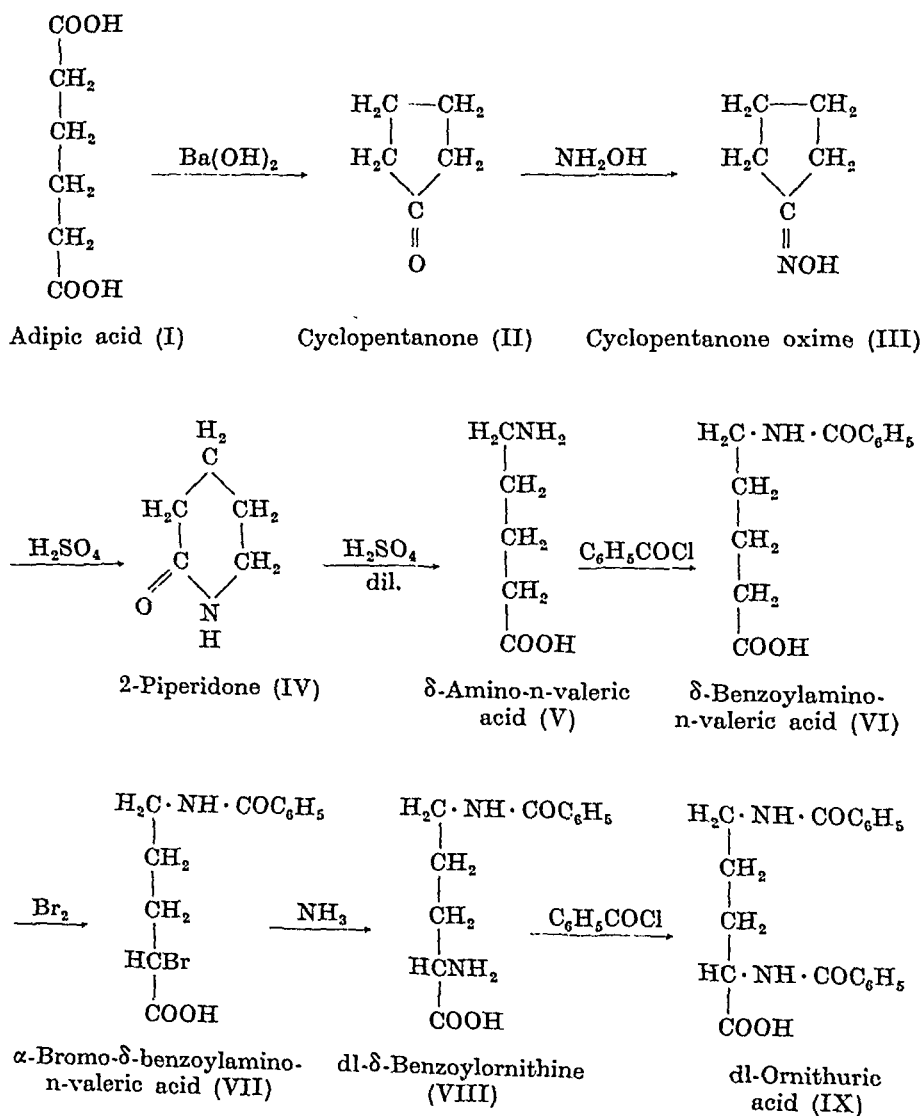
The free base of synthetic dl-lysine, so far as the author is aware, has not previously been produced. For its production the author used the same technique as that proposed by VICKERY and LEAVENWORTH (1928) for the isolation of l-lysine. Broadly speaking, the procedure was that the dl-lysine hydrochloride was ~~treated with silver carbonate in a sulfuric acid solution until all the~~ speaking, the procedure was that the dl-lysine hydrochloride was ~~treated with silver carbonate in a sulfuric acid solution until all the~~ was removed as sulfide and the sulfuric acid was quantitatively precipitated as barium sulfate, the free dl-lysine thus obtained

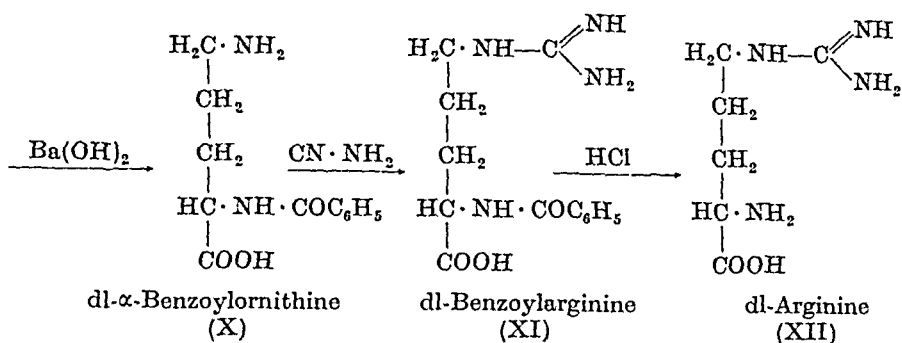
being transferred to warm alcohol and ether added to precipitate the dl-lysine.

For a historical review of the syntheses of the different amino acids, the reader is referred to surveys by SCHMIDT (1945) and DUNN and ROCKLAND (1947).

### dl-Arginine

The synthesis was made in accordance with the following scheme.





I—II. *Cyclopentanone* (II) was produced (THORPE and KON 1946) by heating a mixture of adipic acid and barium hydroxide to 290° C. It was found expedient to use 2000 g adipic acid and 100 g Ba(OH)<sub>2</sub> · 8 H<sub>2</sub>O per batch. The yield of cyclopentanone, b. p., 128—131° C., was 57—77 %.

II—III. *Cyclopentanone oxime* (III). By treating cyclopentanone with hydroxyl amine (FOX, DUNN and STODDARD 1941), cyclopentanone oxime was produced. It was distilled in a vacuum, the boiling point being at 93—97° C. / 24 mm. M. p. 54—55° C.<sup>1</sup>

The yield was 58—72 %.

III—VI. *δ-Benzoylamino-n-valeric acid* (VI). This synthesis was made according to FOX, DUNN, and STODDARD (*loc. cit.*). Cyclopentanone oxime was converted into piperidone by heating with 30 N H<sub>2</sub>SO<sub>4</sub>. After hydrolysis with diluted H<sub>2</sub>SO<sub>4</sub>, δ-amino-n-valeric acid was obtained; it was benzoylated to δ-benzoylamino-n-valeric acid, which was precipitated and washed with propyl ether. Yield 79—93 %. M. p. 90—91° C.

VI—VIII. *dl-δ-Benzoylornithine* (VIII). According to FOX, DUNN, and STODDARD (*loc. cit.*), δ-benzoylamino-n-valeric acid was brominated in carbon tetrachloride in the presence of red phosphorus. The α-bromic acid thus produced (VII) was aminated, whereupon dl-δ-benzoylornithine was obtained. Yield 18—30 %.

VIII—IX. *dl-Ornithuric acid* (IX). The benzoylation of dl-δ-benzoylornithine was performed according to the following method:—

In an enamelled pan holding 15 litres, 500 g dl-δ-benzoylornithine was dissolved in 5 litres of water, 2 kg of ice and 250 ml of 33 % NaOH, with stirring. From a dropping funnel 500 ml benzoyl chloride was added for 30 minutes, the temperature being kept throughout below 10° C. When all the benzoyl chloride had been supplied, the solution was allowed to stand, with stirring, for another hour. The reaction was kept alkaline to phenolphthalein by addition of small portions of 33 % NaOH. The solution was filtered, and concentrated HCl was added until the reaction became highly acid. After 3—4 hours the precipitate was filtered off and washed with 3 portions of 500 ml boiling water and 3 portions of 400 ml ether. The pre-

<sup>1</sup> The melting point was determined with Anschütz' short-stem thermometer.

cipate was allowed to dry in air, whereupon 650 g dl-ornithuric acid (90 %), m. p. 187—188° C., was obtained.

IX—X. *dl- $\alpha$ -Monobenzoylornithine* (X). The dl-ornithuric acid was hydrolyzed with dilute barium hydroxide to dl- $\alpha$ -monobenzoylornithine according to the method of SÖRENSEN, HÖYRUP, and ANDERSEN (1911). Yield 63—74 %; m. p. 264—267° C.

X—XI. *dl- $\alpha$ -Monobenzoylarginine* (XI). By treatment of dl- $\alpha$ -monobenzoylornithine with cyanamide according to the method of SÖRENSEN, HÖYRUP, and ANDERSEN (*loc. cit.*), dl- $\alpha$ -monobenzoylarginine was obtained. Yield 59—80 %; m. p. 310—315° C. (dec.). The cyanamide was produced according to the method of OSTERBERG and KENDALL (1917) from calcium cyanamide and carbon dioxide.

XI—XII. *dl-Arginine HCl* (XII). The dl- $\alpha$ -monobenzoylarginine was hydrolyzed with concentrated HCl according to the method of SÖRENSEN, HÖYRUP, and ANDERSEN (*loc. cit.*); the benzoic acid was filtered off and any residual benzoic acid in the solution was removed by extraction with ether. In order to obtain dl-arginine monohydrochloride, the author utilized the above mentioned technique adopted by Cox (1928) in the production of l-arginine HCl.

55 g dl- $\alpha$ -monobenzoylarginine was dissolved in 250 ml concentrated HCl. The solution was evaporated to dryness on a steam bath, which procedure was once again repeated. The remainder was dissolved in 250 ml concentrated HCl and was refluxed for 6 hours, whereupon the solution was cooled in an ice bath and the precipitated benzoic acid was filtered off. The filtrate was extracted with three portions of 250 ml ether and the aqueous solution was evaporated in a vacuum to a thick syrup, which was dissolved in 150 ml 95 % alcohol. With vigorous stirring, 20 ml aniline was added. Crystallization of dl-arginine HCl was induced with a glass rod. The mixture was placed in the refrigerator over night, whereupon the arginine HCl was filtered off and washed with two portions of 50 ml absolute alcohol.

dl-Arginine HCl was further purified by dissolving it in 150 ml water and boiling the solution for half an hour with 3 g washed activated carbon. After filtration, the filtrate was evaporated in a vacuum to a thin syrup (30 ml), which was cooled to room temperature, and 95 % alcohol was added with stirring until a permanent cloudiness appeared. The walls of the glass beaker were scraped until crystallization set in and 160 ml absolute alcohol was gently added; the solution was kept in the refrigerator over night. The precipitated dl-arginine HCl was filtered off and washed with two portions of 50 ml absolute alcohol and two portions of 50 ml ether. The yield of the dl-arginine HCl was 38 grams.

dl-Arginine HCl was purified once again in the following way. The arginine HCl obtained was dissolved in 50 ml distilled water. After filtration, the arginine HCl was precipitated with 285 ml 96 % alcohol and 285 ml ether, which were added whilst stirring. The solution was kept in the refrigerator over night, whereupon the dl-arginine HCl was filtered off and washed with 3 portions of 30 ml ether. Yield 34 grams (82 %), sintering at



199—202° C and decomposing at 240—245° C. On repeated syntheses in accordance with this method, yield ranging between 57 och 82 % were obtained.

#### ANALYSIS.

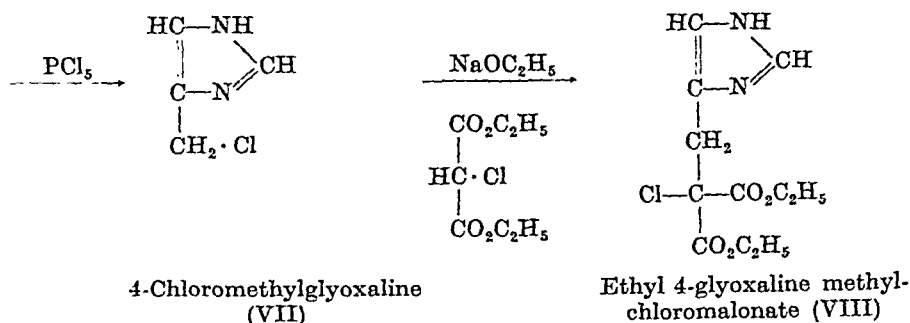
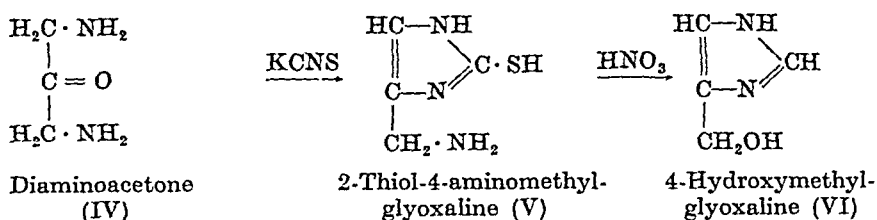
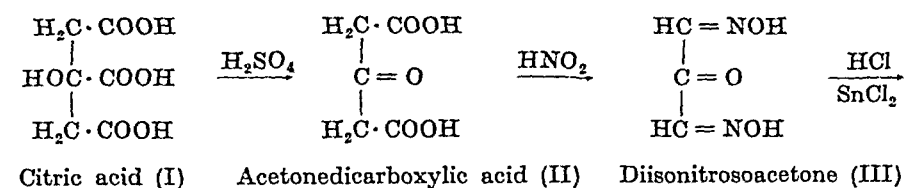
Found.....	C 35.0 %	H 7.20 %	N 26.20 %
C <sub>6</sub> H <sub>11</sub> O <sub>2</sub> N <sub>4</sub> HCl requires..	C 34.19 %	H 7.18 %	N 26.61 %

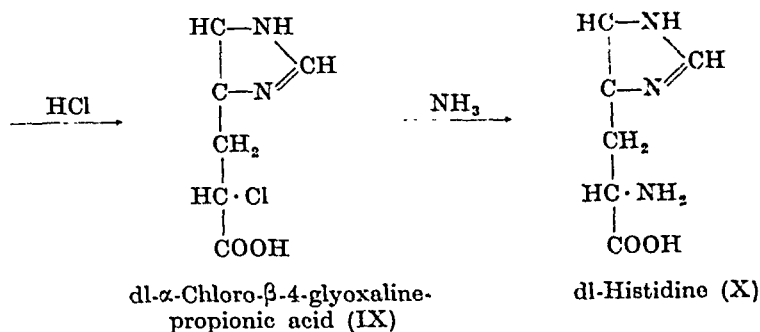
### dl-Histidine

This amino acid was produced firstly according to the method of PYMAN (1911 b) and secondly according to that of ALBERTSON and ARCHER (1945).

#### Synthesis of dl-histidine according to Pyman

In the synthesis of dl-histidine proposed by PYMAN (1911 b), one sets out from citric acid, which was converted into dl-histidine in accordance with the following scheme:—





I—II. *Acetonedicarboxylic acid* (II). Anhydrous citric acid was converted into acetonedicarboxylic acid with fuming sulfuric acid according to v. PECHMAN (1891). The product thus obtained contained ca. 20 % sulfuric acid. No purification was made before its use for the next synthesis. From 12200 grams citric acid, 9620 grams of the said product was obtained.

II—III. *Diisonitrosoacetone* (III). From the crude acetonedicarboxylic acid, diisonitrosoacetone was produced by treatment in cold with sodium nitrite according to v. PECHMAN and WEHSARG (1886). The yield from 9320 grams crude acetonedicarboxylic acid was 1484 grams diisonitrosoacetone, m. p. 143—144° C.

III—IV. *Diaminoacetone, di HCl* (IV). Diisonitrosoacetone was reduced with  $\text{SnCl}_2$  to diaminoacetone, which was precipitated as diaminoacetone di HCl,  $\text{SnCl}_2$ ; the latter was dissolved in hydrochloric acid. With hydrogen sulfide, stannous sulfide was precipitated, whereupon diaminoacetone di HCl was obtained by concentration of the filtrate. The method was that proposed by KALISCHER (1895). Yield 52—65 %.

IV—VI. *4-Hydroxymethylglyoxaline HCl* (VI). The following new method was elaborated for the preparation of 4-hydroxymethylglyoxaline HCl.

In a 2-litre beaker 210 g potassium thiocyanate was dissolved in 300 ml hot water and was put on a boiling water bath. 300 g diaminoacetone di HCl was added. In the clear solution a precipitate was formed after a few minutes. The reaction mixture was kept for 1½ hour on the water bath and then cooled to room temperature. The precipitate was filtered off, boiled with 450 ml water and filtered hot.

The filtrates thus obtained were mixed together and poured gently down into 3000 ml of 6 % nitric acid, which was kept boiling. After the addition the boiling was continued for another 10 minutes, whereupon the solution was neutralized with 33 % sodium hydroxide and poured into a hot solution of 280 g picric acid in 6000 ml of water. The solution was left over night at room temperature. The crystallized picrate of the 4-hydroxymethylglyoxaline was filtered off and washed in 2 portions of 100 ml cold water. The picrate was recrystallized from the 6000 ml water. The precipitate was washed with 2 portions of 100 ml cold water and, after drying in air, weighed 370 grams; m. p. 203—206° C (dec.)

370 grams 4-hydroxymethylglyoxaline picrate was suspended in 3000 ml 7 % HCl and the picric acid extracted with ether, whereupon the hy-

droxymethylglyoxaline entered into solution as hydrochloride. The solution was evaporated to dryness in a vacuum, extracted with 300 ml warm absolute alcohol, filtered and condensed on a water bath to a thick oil, from which 4-hydroxymethylglyoxaline HCl crystallized on cooling. The crystals were filtered off and washed twice with 100 ml ether. Yield 90—105 grams (36—42 %); m. p. 107—109° C.

VI—VII. *4-Chloromethylglyoxaline HCl* (VII). 4-Hydroxymethylglyoxaline HCl was converted into the corresponding chloromethyl compound (VII) with phosphorous pentachloride according to the method of PYMAN (1911 a). Yield 71—87 %; m. p. 140—142° C.

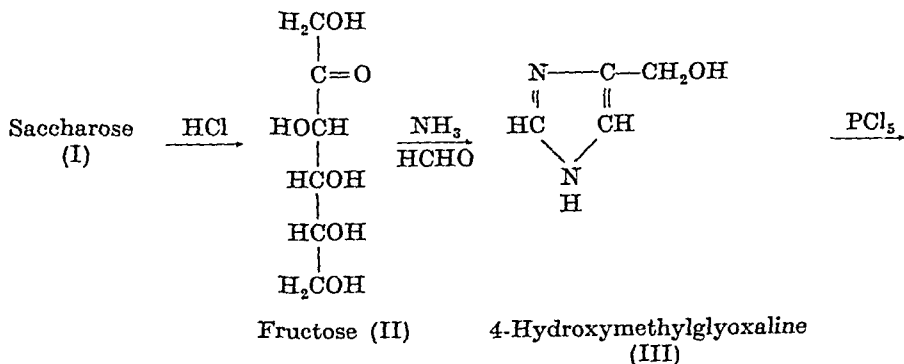
VII—VIII. *Ethyl 4-glyoxalinemethylchloromalonate HCl* (VIII). This synthesis was made according to the method of PYMAN (1911 b), the 4-chloromethylglyoxaline being condensed with ethyl chloromalonate in the presence of sodium ethoxide to ethyl 4-glyoxalinemethylchloromalonate. The hydrochloride of the latter was isolated via the oxalate salt. Yield 20—27 %; m. p. 148—149° C.

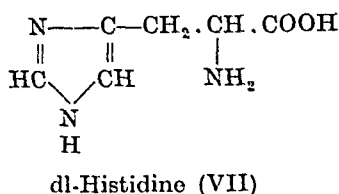
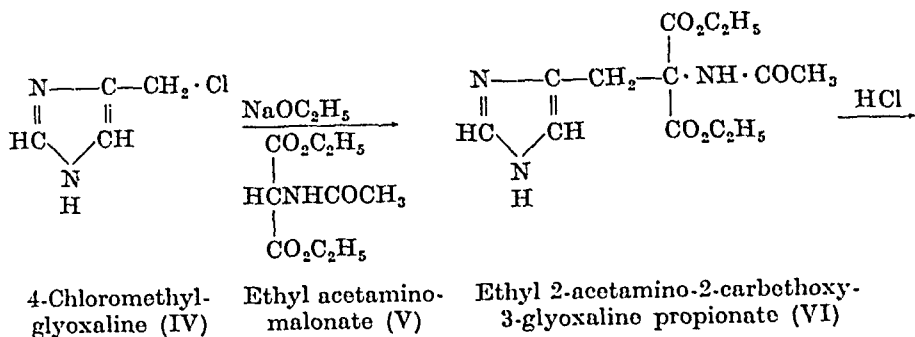
VIII—IX. *dl-α-Chloro-β-4-glyoxalinepropionic acid* (IX). By hydrolysis, decarboxylation of (VIII) by boiling with HCl according to the method of PYMAN (1911 b) and removal of HCl with silver oxide, dl-α-chloro-β-4-glyoxalinepropionic acid was obtained. Yield 85—90 %; m. p. 201° C.

IX—X. *dl-Histidine HCl, 2H<sub>2</sub>O*. According to PYMAN (*loc. cit.*), dl-α-chloro-β-4-glyoxalinepropionic acid was aminated with ammonia under pressure. After recrystallization from water and drying in air, the substance had a melting point of 117—119° C. Yield 35—40 %. The dl-histidine HCl, 2H<sub>2</sub>O was converted to the free base dl-histidine according to the aniline-method described on page 28.

### Production of dl-histidine according to Albertson and Archer

The synthesis of dl-histidine by the method of ALBERTSON and ARCHER (1945) was effected in accordance with the following scheme:—





I—III. *4-Hydroxymethylglyoxaline HCl* (III). This synthesis was effected from fructose according to the procedure of TOTTER and DARBY (1944). The fructose was obtained by hydrolysis of saccharose (ALBERTSON and ARCHER *loc. cit.*). In a solution of formaldehyde,  $\text{NH}_3$  and  $\text{CuCO}_3$ ,  $\text{Cu(OH)}_2$ , the fructose was converted into a sparingly soluble copper complex of 4-hydroxymethylglyoxaline. After removal of the copper as sulfide, the 4-hydroxymethylglyoxaline was purified via the picrate. The picric acid was removed and the hydroxymethylglyoxaline HCl was precipitated from an alcoholic solution with ether. Yield 36—43 %, estimated as though a molecule of fructose or saccharose gave a molecule of 4-hydroxymethylglyoxaline; m. p. 107—109° C.

III—IV. *4-Chloromethylglyoxaline HCl* (IV). The synthesis was made according to the method described on p. 26.

V. *Ethyl acetaminomalonate* (V). The synthesis was carried out in the following manner:—

A 3-litre beaker with 400 g ethyl malonate in 450 g acetic acid was cooled in an ice bath. A solution of 518 g sodium nitrite in 710 ml water was added whilst stirring, care being taken that the temperature of the solution was kept below +20° C. The mixture was stirred for another four hours. The reaction mixture was extracted with three 500 ml portions of ether. The combined ether extracts were extracted with three 250 ml portions of 10 %  $\text{Na}_2\text{CO}_3$  and two 250 ml portions of water. The ether solution was dried by means of calcium chloride and after filtration the ether was distilled off in a vacuum. The residue was dissolved in 1000 ml absolute alcohol, and 25 g 10 % Pd-charcoal catalyst was added. The mixture was hydrogenated at a pressure of 150 kg/cm<sup>2</sup> and the temperature was raised to 130—150° C; when the absorption of hydrogen had ceased, the Pd-charcoal was filtered off and the alcohol was distilled off in a vacuum; the residue was dissolved in 1 litre of ether, to which 204 g acetic anhydride was added whilst stirring,

so that the ether boiled quietly. On cooling, white crystals of ethyl acetaminomalonate precipitated and were filtered off; yield 175—240 g (32—44 %), m. p. 95—96° C.

V—VI. *Ethyl 2-acetamino-2-carbethoxy-3-glyoxalinepropionate* (VI). By condensation of ethyl acetaminomalonate and 4-chloromethylglyoxaline in sodium ethoxide-alcohol, ethyl 2-acetamino-2-carbethoxy-3-glyoxalinepropionate was obtained according to the technique proposed by ALBERTSON and ARCHER (*loc. cit.*). It should be noted that the temperature of the sodium ethoxide-alcohol should be between + 5 and + 10° C. when the ethyl acetaminomalonate is added. The product was purified by recrystallization in water and then had a melting point of 96—97° C. Yield 50—65 %.

VI—VII. *dl-Histidine* (VII). By hydrolysis and decarboxylation of ethyl 2-acetamino-2-carbethoxy-3-glyoxalinepropionate with hydrochloric acid, *dl*-histidine was obtained and was isolated according to ALBERTSON and ARCHER (*loc. cit.*) as dihydrochloride. In order to obtain the free base *dl*-histidine, the author devised the following procedure:—

155 g ethyl 2-acetamino-2-carbethoxy-3-glyoxalinepropionate was refluxed with 2000 ml concentrated HCl in a 4-litre flask for 6 hours. The solution was concentrated by evaporation in a vacuum into a thick syrup, which was mixed with warm 5 N HCl to a volume of 125 ml, whereupon 500 ml absolute alcohol was added, with stirring. The mixture was kept in the refrigerator over night; the precipitate was filtered off and washed with 2 portions of 50 ml absolute alcohol. The precipitate, which consisted of *dl*-histidine di HCl, was dissolved in 900 ml boiling water, 110 g aniline was added whilst stirring, whereupon 900 ml absolute alcohol was supplied. The mixture was kept in the refrigerator over night, the precipitate of impure *dl*-histidine thus obtained was filtered off and washed with two portions of 100 ml absolute alcohol.

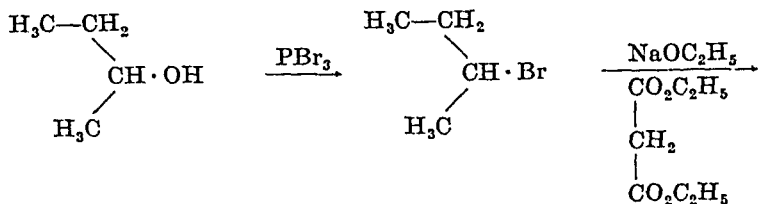
The impure *dl*-histidine was dissolved in 700 ml of hot water and 25 % ammonia was added so that the solution had a pH of 7.6; the solution was filtered warm, whereupon 700 ml of warm absolute alcohol was added, and the mixture was kept in the refrigerator over night. The precipitate of pure crystalline *dl*-histidine was filtered off and washed with 2 portions of 50 ml absolute alcohol and 2 portions of 50 ml ether. After drying in air, 34—40 g *dl*-histidine (42—50 %) was obtained; m. p. 284—285° C (dec.).

#### ANALYSIS.

Found.....	C 46.36 %	H 5.83 %	N 27.00 %
C <sub>6</sub> H <sub>9</sub> O <sub>2</sub> N <sub>3</sub> requires .....	C 46.42 %	H 5.85 %	N 27.10 %

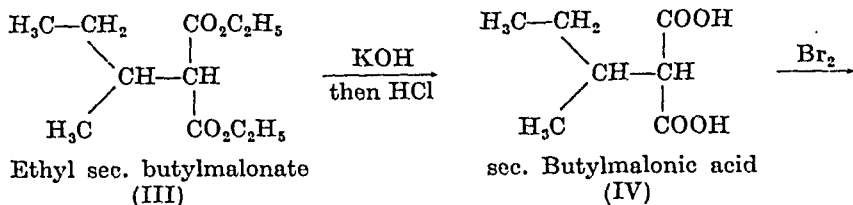
#### *dl*-Isoleucine

This amino acid was produced in accordance with the following scheme, described by MARVEL (1941 a).



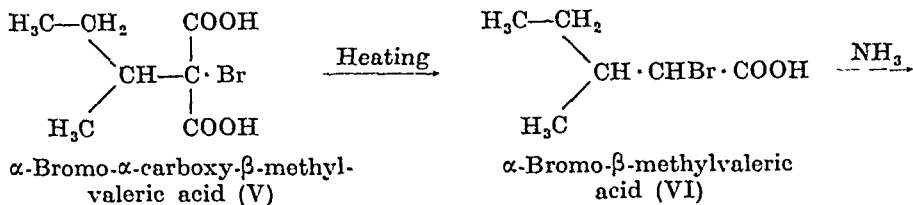
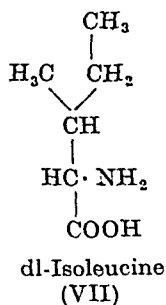
sec. Butyl alcohol (I)

sec. Butyl bromide (II)



Ethyl sec. butylmalonate (III)

sec. Butylmalonic acid (IV)

 $\alpha$ -Bromo- $\alpha$ -carboxy- $\beta$ -methylvaleric acid (V) $\alpha$ -Bromo- $\beta$ -methylvaleric acid (VI)

dl-Isoleucine (VII)

I—II. *sec. Butyl bromide* (II) was produced from *sec. butyl alcohol* b. p. 99.5° C., and bromine in the presence of phosphorus, according to the method of GOSHORN and BOYD (1946). Yield 50—60 %; b. p. 90—93° C.

II—III. *Ethyl sec. butylmalonate* (III). In sodium ethoxide-alcohol, *sec. butyl bromide* and *ethyl malonate* were condensed according to the method of MARVEL (*loc. cit.*). Yield 80—85 %, b. p. 110—120° C. / 18—20 mm.

III—VI.  $\alpha$ -Bromo- $\beta$ -methylvaleric acid (VI). In accordance with the technique of MARVEL (*loc. cit.*), *ethyl sec. butyl malonate* was hydrolyzed with KOH, in which process the potassium salt of the acid (IV) was obtained. After acidification the free acid (IV) was extracted with ether. *sec. Butylmalonic acid* was brominated in the ether solution to  $\alpha$ -bromo- $\alpha$ -carboxy- $\beta$ -methylvaleric acid, which was decarboxylated by heating, and the  $\alpha$ -bromo- $\beta$ -methylvaleric acid obtained was distilled at 125—140° C. / 18—20 mm. Yield 60—65 %.

VI—VII. *dl*-Isoleucine (VII). By amination of the bromic acid (VI) with ammonia according to the method of MARVEL (*loc. cit.*), *dl*-isoleucine was then obtained. Yield 34—40 %; m. p. 278—280° C. (dec.). The *dl*-isoleucine recrystallized in conformity with Marvel's technique was further purified by recrystallization in 30 % alcohol in the following manner:—

600 g *dl*-isoleucine was dissolved in 7400 ml of boiling water. The solution was filtered warm, mixed with 3200 ml of absolute alcohol and kept in the refrigerator over night. The precipitate was filtered off and washed with two portions of 400 ml absolute alcohol and 1 litre of water. After drying in air, the weight was 460—480 grams; m. p. 278—280° C. (dec.).

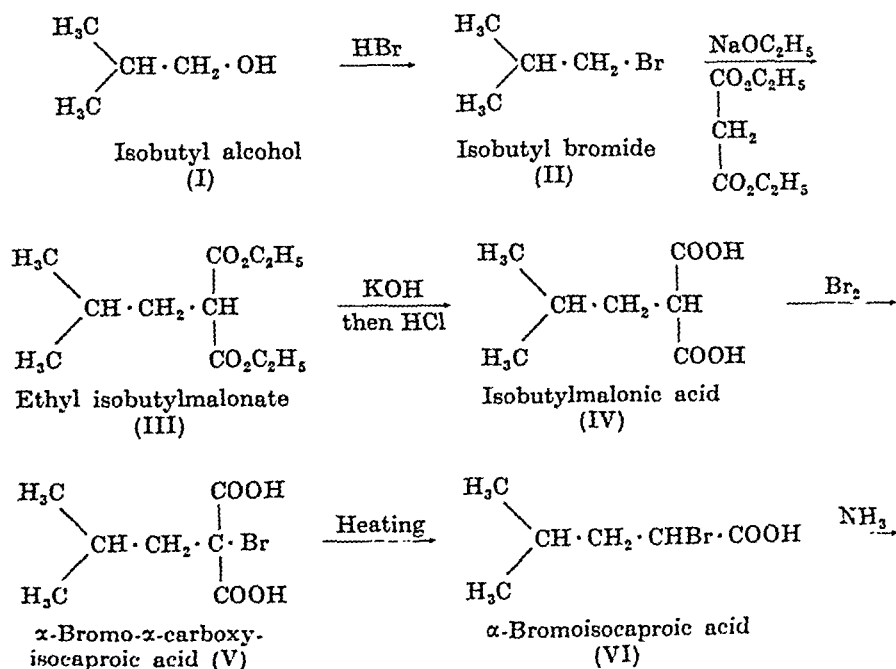
#### ANALYSIS.

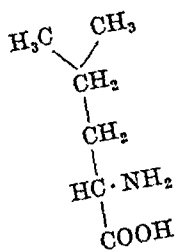
Found.....	C 54.87 %	H 9.92 %	N 10.67 %
$C_6H_{13}O_2N$ .....	C 54.92 %	H 9.99 %	N 10.68 %

#### *dl*-Leucine

For the synthesis of *dl*-leucine the following method was devised. As mentioned above, the procedure was based on a method of FISCHER and SCHMITZ (1906). The procedure is analogous with that adopted by MARVEL (1941 a) for the synthesis of *dl*-isoleucine.

The syntheses were carried out in accordance with the following scheme:—





dl-Leucine (VII)

I—II. Isobutyl bromide (II) was produced from isobutyl alcohol, b. p. 07—108° C., according to the method of BODROUX (1915). Yield 50—55 %  
 d. p. 90—92° C.

II—III. Ethyl isobutylmalonate (III) was obtained by condensation of ethyl malonate and isobutyl bromide with sodium ethoxide in alcohol according to the following method:—

Into a 12-litre three-necked flask having a reflux condenser with a CaCl<sub>2</sub> seal, a stirrer with a mercury seal and a dropping funnel, 4900 ml absolute alcohol and 245 g metallic sodium were introduced. When all the sodium had completely reacted, 1750 g ethyl malonate was added and afterwards 1470 g isobutyl bromide through the dropping funnel at such a rate that the alcohol boiled quietly. The mixture was kept on a water bath for 48 hours with stirring, whereupon the alcohol was distilled off and the residue was mixed with 1400 ml water. The oil layer (ethyl isobutylmalonate) thus produced was separated and distilled in a vacuum, at 115—125° C. / 20 mm. The yield of ethyl isobutylmalonate was 1600—1700 grams (67—71 %).

III—VI.  $\alpha$ -Bromoisocaproic acid (VI). Ethyl isobutylmalonate was saponified with KOH, and the free acid (IV) was extracted with ether after the addition of hydrochloric acid. The acid (IV) was brominated in the ether solution, and  $\alpha$ -bromo- $\alpha$ -carboxyisocaproic acid was thus obtained. The latter product was decarboxylated by heating to  $\alpha$ -bromoisocaproic acid, which was afterwards distilled in a vacuum. For this synthesis the following method was adopted:—

In a 30-litre enamelled pan a solution of 3250 g KOH in 2600 ml of water was heated to 100° C., whereupon 3250 g ethyl isobutylmalonate was slowly supplied, with stirring. The solution was kept at 100—110° C. for 5 hours, water being supplied in sufficient amount to prevent drying. The solution was cooled in a water bath, and concentrated hydrochloric acid was added until the solution became strongly acid. In this latter process the temperature must not exceed +20° C. The free dicarbonic acid (IV) was extracted with three portions of 2600 ml ether. The total ether solution was dried with calcium chloride over night. After filtration the ether solution was introduced into a three-necked 12-litre flask with a reflux condenser, a stirrer with a mercury seal and a dropping funnel. With stirring, 60 ml bromine was added and, when the solution had lost its colour, a further amount of 650 ml bromine was added at such a rate that the ether boiled steadily. When all the bromine had been added, 2600 ml of water was



supplied, at first gently. The ether solution was separated from the water, and the ether distilled off. The residue, which consisted of  $\alpha$ -bromo- $\alpha$ -carboxyisocaproic acid, was refluxed for 5 hours in a 6-litre flask on an oil bath heated to  $130^{\circ}\text{C}$ ., whereupon the  $\alpha$ -bromoisocaproic acid was distilled in a vacuum and the fraction between  $125$  and  $130^{\circ}\text{C}/12\text{ mm}$  was taken. Yield 1900—2000 grams (65—68 %).

VI—VII. *dl*-Leucine (VII). The amination of  $\alpha$ -bromoisocaproic acid was effected by treatment with ammonia according to the method indicated by MARVEL (1941 b). Yield 38—45 %; m. p.  $290$ — $295^{\circ}\text{C}$ . (dec.).

The *dl*-leucine thus obtained was recrystallized once again, 490 g being dissolved in 10 litres of boiling hot water, and 7 litres of warm absolute alcohol being added. The solution was kept in the refrigerator over night, the precipitate of *dl*-leucine was filtered off and washed with 500 ml of 50 % alcohol, 500 ml of absolute alcohol and two portions of 800 ml ether. The filtrate was evaporated in a vacuum to 2 litres, whereupon the solution was warmed and filtered; after the addition of 1 litre of 95 % alcohol, it was placed in the refrigerator over night. The crystalline *dl*-leucine was filtered off and washed with 200 ml of 50 % alcohol, 200 ml of absolute alcohol and 200 ml of ether. Yield 450 grams; m. p.  $290$ — $293^{\circ}\text{C}$ . (dec.).

#### ANALYSIS.

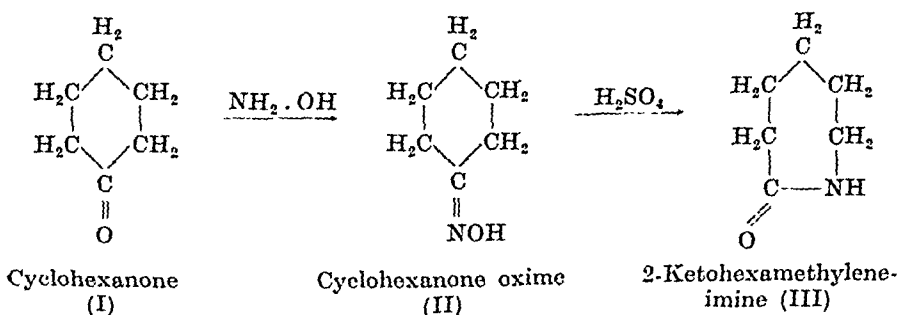
Found.....	C 54.95 %	H 10.05 %	N 10.67 %
$\text{C}_6\text{H}_{13}\text{O}_2\text{N}$ requires.....	C 54.92 %	H 9.99 %	N 10.68 %

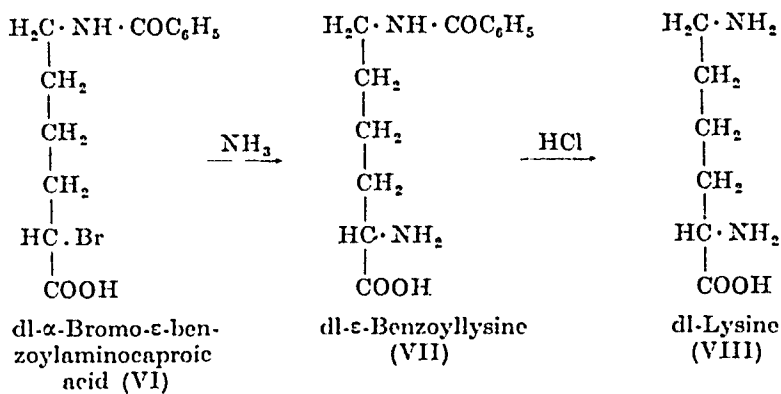
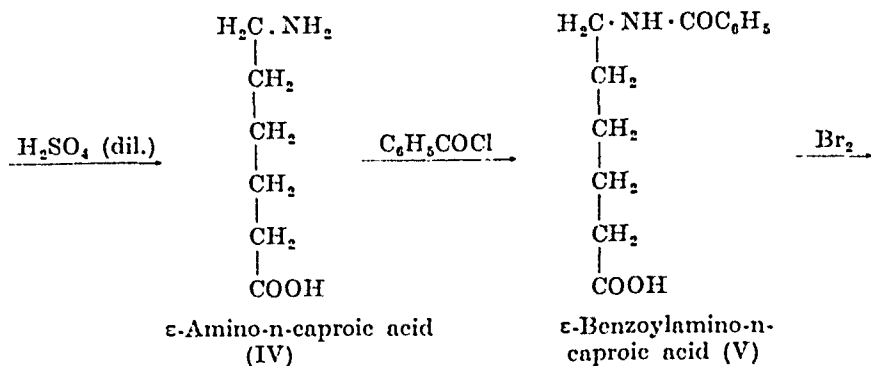
#### *dl*-Lysine

This amino acid was produced 1) as *dl*-lysine monohydrochloride and 2) as the free base *dl*-lysine.

The method for the production of the free base *dl*-lysine has been elaborated by the author. It was analogous with VICKERY and LEAVENWORTH's process (1928) for the production of the free base *l*(+)-lysine.

The procedure for the syntheses is shown in the following scheme:—





I—II. *Cyclohexanone oxime* (II) was produced from cyclohexanone and hydroxylamine according to the technique proposed by BOUSQUET (1946). Cyclohexanone oxime was distilled in a vacuum at 100—105° C. / 10—12 mm. Yield 80—90 %; m. p. 86—88° C.

II—V. *ε-Benzoylamino-n-caproic acid* (V) was produced according to the technique of ECK and MARVEL (1946, b) from cyclohexanone oxime, which, by heating with 85 % sulfuric acid, was converted into 2-ketohexamethyleneimine. The solution thus obtained was diluted with water and boiled, the 2-ketohexamethyleneimine being hydrolyzed to *ε*-amino-n-caproic acid. After benzoylation, *ε*-benzoylamino-n-caproic acid was obtained. Yield 73—82 %; m. p. 77—80° C.

V—VI. *dl-α-Bromo-ε-benzoylamino-ε-caproic acid* (VI). According to the technique proposed by ECK and MARVEL (1946 a), *ε*-benzoylamino-n-caproic acid was converted, by treatment with bromine in the presence of red phosphorus, into *dl-α*-bromo-*ε*-benzoylamino-n-caproic acid. Yield 70—75 %; m. p. 162—165° C.

VI—VII. *dl-ε-Benzoyllysine* (VII). By treatment of *dl-α*-bromo-*ε*-benzoylamino-n-caproic acid with ammonia, *dl-ε*-benzoyllysine according to the technique of ECK and MARVEL (1946 c) was obtained. Yield 35—43 %; m. p. 265—270° C.

VII—VIII. *dl-Lysine HCl* (VIII). *dl-ε*-Benzoyllysine was hydrolyzed with hydrochloric acid and *dl*-lysine di-HCl was isolated. This was con-

verted in pyridine-alcohol to dl-lysine HCl. The latter was dissolved in water and was precipitated with alcohol. In this synthesis ECK and MARVEL's technique (1946 c) was adopted. The product obtained was dl-lysine HCl with a melting point of 263—264° C. Yield 55—65 %.

Further purification of dl-lysine HCl was effected in the following way:

90 g dl-lysine HCl was dissolved in 180 ml boiling water and boiled for half an hour with 3 g washed activated carbon. The mixture was filtered warm and 1.4 litre of warm 95 % alcohol was added whilst stirring. The solution was placed, for crystallization, in the refrigerator over night; the precipitate was sucked off and washed with 2 portions of 100 ml 95 % alcohol and 3 portions of 100 ml ether. Yield 95—100 %; m. p. 263—264° C.

#### ANALYSIS.

Found.....	C 39.45 %	H 8.26 %	N 15.30 %
$C_6H_{14}O_2N_2$ HCl requires .	C 39.46 %	H 8.28 %	N 15.34 %

IX. *dl-Lysine*. For the production of the free base dl-lysine from dl-lysine HCl, the following method was adopted:—

120 g dl-lysine HCl was dissolved in 1000 ml water. This solution was mixed with silver carbonate (precipitated from 170 g  $AgNO_3$ , dissolved in 600 ml water and 60 g  $Na_2CO_3$  in 400 ml water; the precipitate was washed with 4 portions of 500 ml distilled water). Thereupon 5 N sulfuric acid was supplied, so that the mixture was throughout acid. The mixture was shaken and was allowed to stand until a filtrate no longer gave any chloride reaction with a silver nitrate solution. The mixture was filtered and the precipitate was washed with two portions of 200 ml hot water; the filtrates were then treated with hydrogen sulfide until all the silver had been precipitated, whereupon the silver sulfide was filtered off and washed with two portions of 100 ml hot water. The solution was evaporated to 1000 ml, boiled for 10 minutes with 5 g washed activated carbon and filtered. To the filtrate a saturated barium hydroxide solution was added in such an amount that all the sulfuric acid was precipitated, care being taken that there was no excess of barium hydroxide. The barium sulfate was filtered off and washed with two portions of 200 ml boiling water. The filtrates were evaporated to dryness, whereupon the dl-lysine was dissolved in 1000 ml warm absolute alcohol, was filtered, and 1000 ml ether was added to the filtrate, the result being the precipitation of dl-lysine. The precipitate was washed with two portions of 200 ml ether; after drying in air, it weighed 80 grams (83 %); m. p. 164—166° C.

#### ANALYSIS.

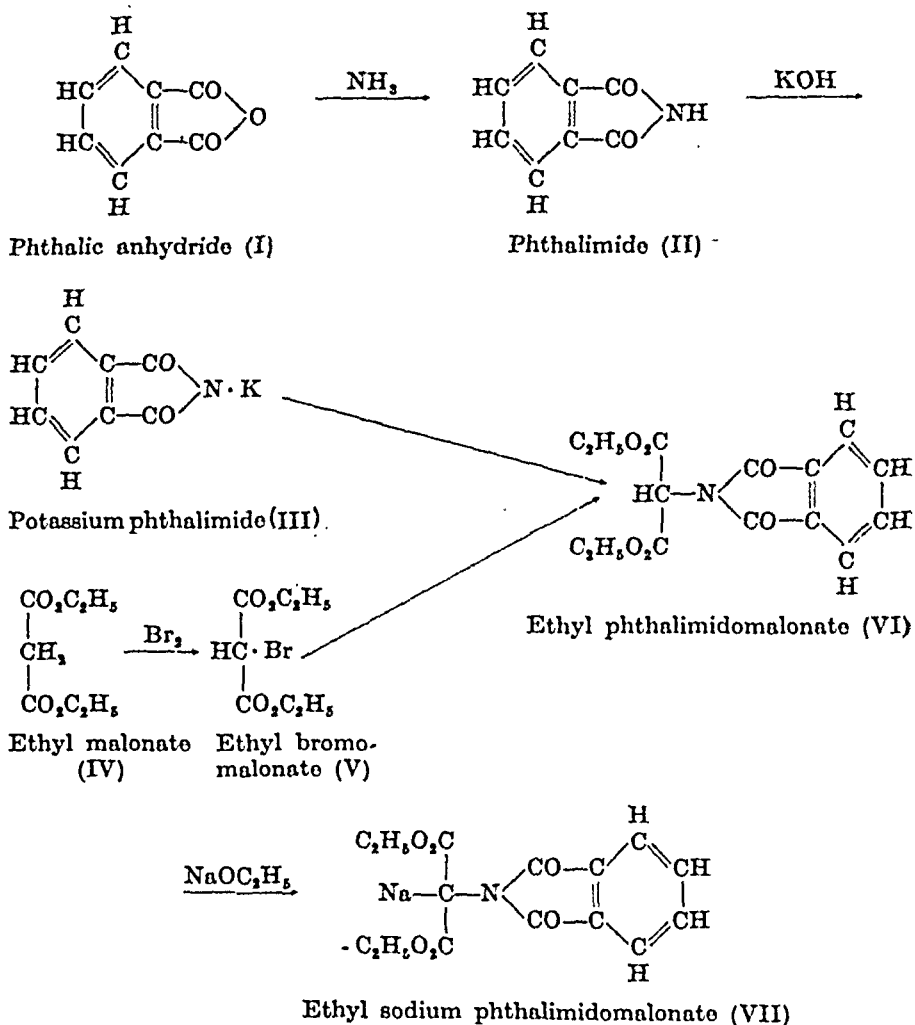
Found.....	C 49.0 %	H 10.0 %	N 19.22 %
$C_6H_{14}O_2N_2$ requires ....	C 49.27 %	H 9.66 %	N 19.17 %

### dl-Methionine

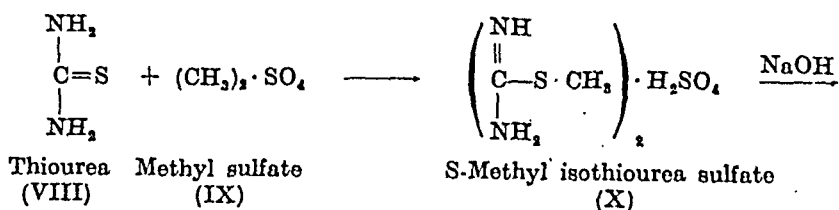
dl-Methionine was produced according to the technique of BARGER and WEICHSELBAUM (1946) from ethyl sodium phthalimido-

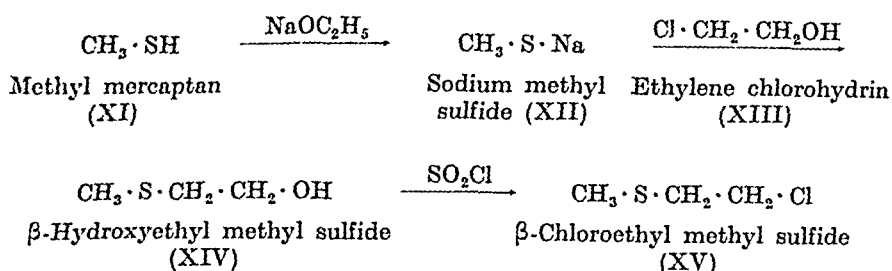
malonate and  $\beta$ -chloroethyl methyl sulfide. Thus, before this synthesis the two latter substances must be produced.

In the synthesis of ethyl sodium phthalimidomalonate, the following reaction scheme was followed:—

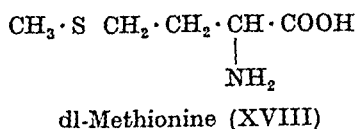
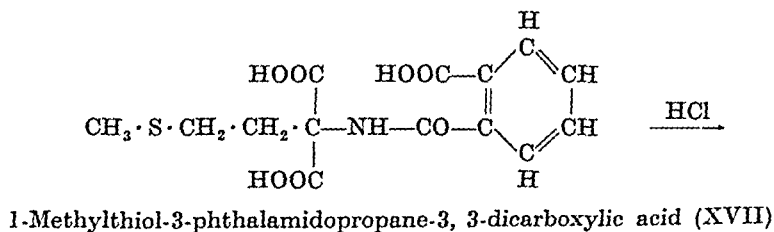
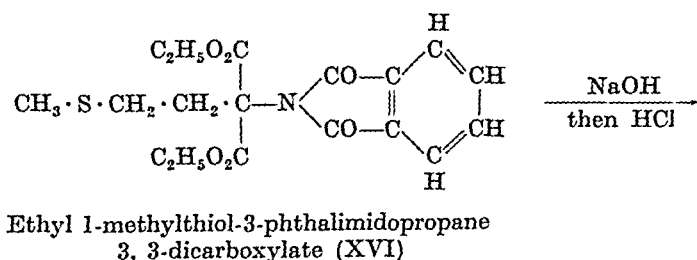
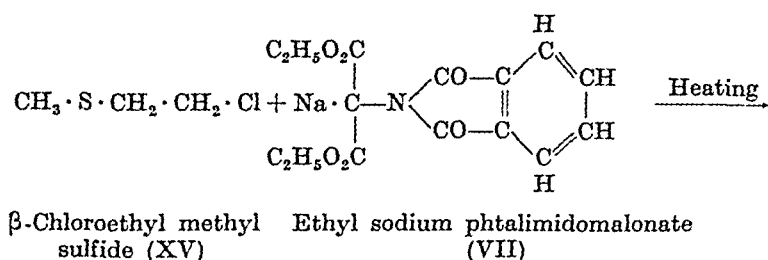


$\beta$ -Chloroethyl methyl sulfide was prepared according to the following scheme:—





The synthesis of dl-methionine was then made from  $\beta$ -chloroethyl methyl sulfide and ethyl sodium phthalimidomalonate according to the method of BARGER and WEICHSELBAUM (1946). The scheme of reaction was the following:—



I—II. *Phthalimide* (II) was produced according to the technique of NOYES and PORTER (1946) by heating phthalic anhydride and ammonia. Yield 93—96 %; m. p. 232—235° C.

II—III. *Potassium phthalimide* (III) was obtained by the treatment of phthalimide with KOH in an alcohol-water solution according to the method of SALZBERG and SUPNIEWSKI (1946). Yield 69—78 %.

IV—V. *Ethyl bromomalonate* was produced by bromination of ethyl malonate in carbon tetrachloride according to the technique indicated by PALMER and MCWHERTER (1946). The ethyl bromomalonate obtained was distilled in a vacuum. Yield 62—72 %; b. p. 121—125° C. / 16 mm.

VI. *Ethyl phthalimidomalonate* (VI) was synthesized by heating a mixture of ethyl bromomalonate and potassium phthalimide according to the method of OSTERBERG (1946). Yield 52—60 %; m. p. 73—74° C.

VII. *Ethyl sodium phthalimidomalonate* (VII) was synthesized according to the method of BARGER and WEICHSELBAUM (1946) from sodium ethoxide and ethyl phthalimidomalonate. Yield 80—85 %.

VIII—X. *S-Methyl isothiurea sulfate* (X). By heating thiourea and dimethyl sulfate in a little water, methyl isothiurea sulfate was obtained according to SCHILDNECK and WINDUS (1946). Yield 78—90 %; m. p. 230—235° C. (dec.).

X—XIV.  *$\beta$ -Hydroxyethyl methyl sulfide* (XIV). This synthesis was carried out according to the method of WINDUS and SCHILDNECK (1946) from sodium methyl sulfide and ethylene chlorohydrin in alcoholic solution. Sodium methyl sulfide was produced by introducing methyl mercaptan, obtained by the treatment of methyl isothiurea sulfate with NaOH into sodium ethoxide alcohol. The resulting solution of sodium methyl sulfide was mixed with ethylene chlorohydrin,  $\beta$ -hydroxyethyl methyl sulfide being thus obtained. It was distilled in a vacuum at 68—70° C. / 20 mm. Yield 65—70 %.

XIV—XV.  *$\beta$ -Chloroethyl methyl sulfide* (XV) was prepared from  $\beta$ -hydroxyethyl methyl sulfide and thionyl chloride according to KIRNER and WINDUS (1946). Yield 78—84 %; b. p. 55—56° C. / 30 mm.

XVI. *Ethyl 1-methylthiol-3-phthalimidopropane-3,3-dicarboxylate* (XVI). By heating  $\beta$ -chloroethyl methyl sulfide and ethyl sodium phthalimidomalonate to 160—165° C. according to the method of BARGER and WEICHSELBAUM (*loc. cit.*), ethyl 1-methylthiol-3-phthalimidopropane-3,3-dicarboxylate was obtained. Yield 80—83 %; m. p. 66—67° C.

XVI—XVII. *1-Methylthiol-3-phthalamidopropane-3,3-dicarboxylic acid* (XVII). Ethyl 1-methylthiol-3-phthalimidopropane-3,3-dicarboxylate was treated with sodium hydroxide (BARGER and WEICHSELBAUM *loc. cit.*), the sodium salt of 1-methylthiol-3-phthalamidopropane-3,3-dicarboxylic acid being thus obtained. By acidification, the free acid was then precipitated. Yield 89—95 %; m. p. 141—143° C.

XVII—XVIII. *dl-Methionine* (XVIII). By heating 1-methylthiol-3-phthalamidopropane-3,3-dicarboxylic acid with hydrochloric acid, we obtained by hydrolysis and decarboxylation according to the method of BARGER and WEICHSELBAUM (*loc. cit.*) dl-methionine, which was crystallized from alcohol-pyridin. Yield 80—85 %; m. p. 279—280° C.

Further recrystallization of dl-methionine was carried out in the following way:—

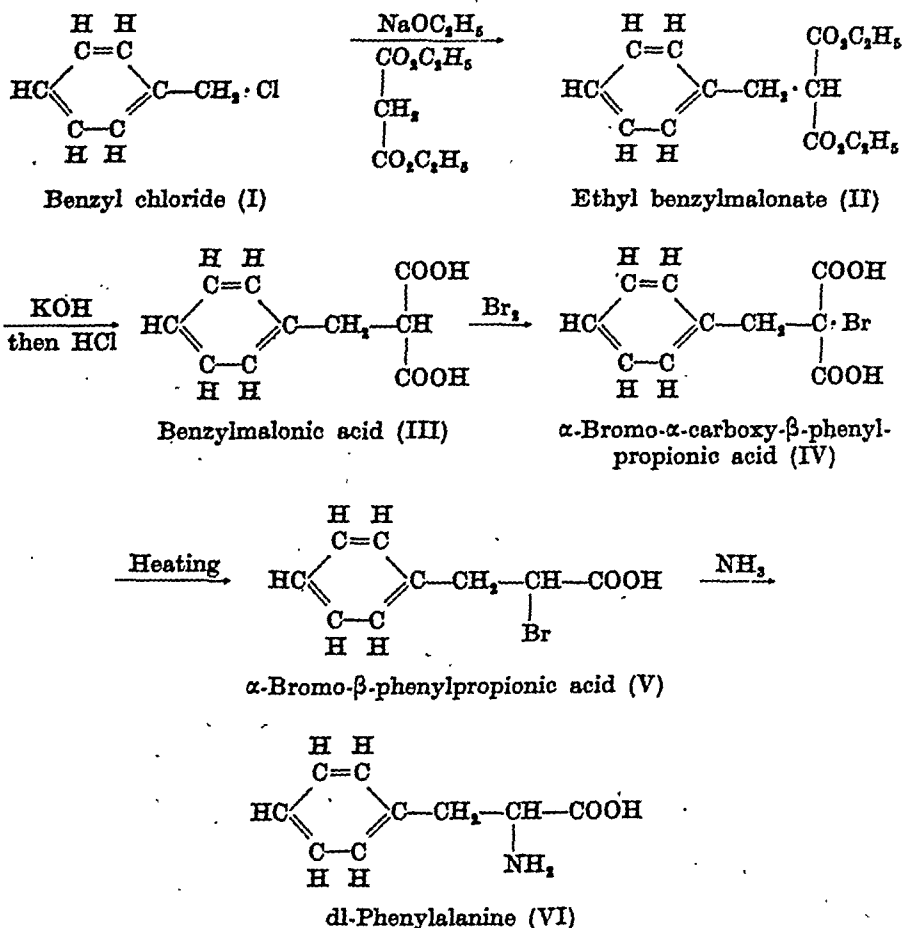
250 g dl-methionine was dissolved in 1500 ml of boiling distilled water: the solution was boiled with 5 g washed activated carbon for half an hour and was then filtered warm. The filtrate was mixed with 4500 ml of warm absolute alcohol and the mixture was placed in the refrigerator over night. The crystals were filtered off and washed with 500 ml absolute alcohol and 1000 ml ether. After drying in air, the weight was 225 grams (90 %) m. p. 279—280° C.

#### ANALYSIS.

Found.....	C 40.80 %	H 7.40 %	N 9.31 %
C <sub>9</sub> H <sub>11</sub> O <sub>2</sub> NS requires.....	C 40.23 %	H 7.43 %	N 9.39 %

### dl-Phenylalanine

dl-Phenylalanine was prepared according to the technique described by MARVEL (1941 c). The synthesis was made in accordance with the following scheme:—



I—II. *Ethyl benzylmalonate* (II) was obtained according to the method of MARVEL (1941 c) by condensation of benzyl chloride and ethyl sodium malonate and was distilled in a vacuum. Yield 52—56 %; b.p. 145—155° C. / 5 mm.

II—VI. *dl-Phenylalanine* (VI). In accordance with the method of MARVEL (*loc. cit.*), ethyl benzylmalonate was hydrolyzed with potassium hydroxide and, after acidification with hydrochloric acid, the free acid (III) was extracted with ether and was brominated in the ether solution. The ether was distilled off, whereupon the residue, consisting of  $\alpha$ -bromo- $\alpha$ -carboxy- $\beta$ -phenylpropionic acid was decarboxylated by heating to  $\alpha$ -bromo- $\beta$ -phenylpropionic acid. This substance was aminated by ammonia. After concentration by evaporation and recrystallization, *dl*-phenylalanine was thus obtained. Yield 25—30 %; m.p. 271—273° C. (dec.).

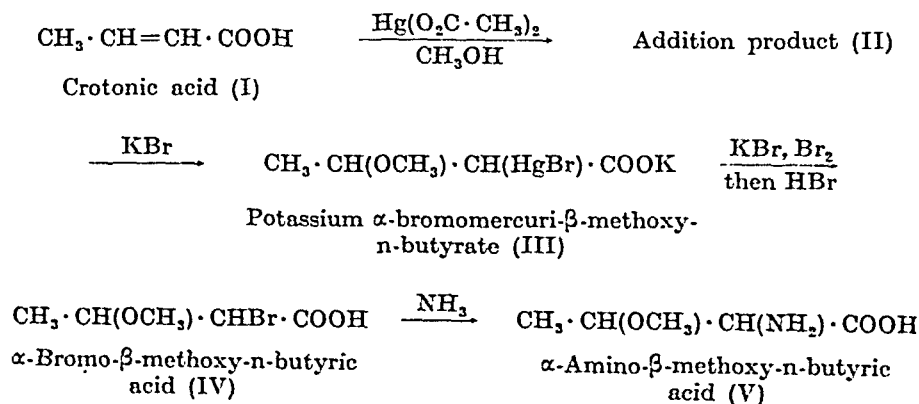
The *dl*-phenylalanine was recrystallized once again in the following way:—  
450 g *dl*-phenylalanine was dissolved in 9 litres of boiling distilled water. The solution was filtered warm and 3 litres of warm absolute alcohol was added, whereupon the mixture was placed in the refrigerator over night. The crystals were filtered off and washed with 400 ml 95 % alcohol, 400 ml absolute alcohol and 1000 ml ether. Yield 380 g *dl*-phenylalanine (83 %); m.p. 271—273° C. (dec.).

#### ANALYSIS.

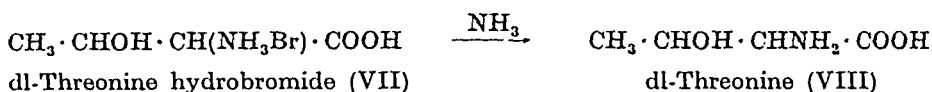
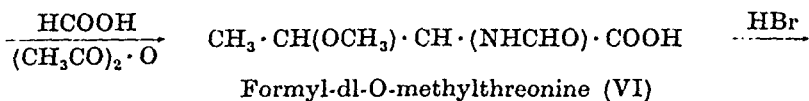
Found.....	C 65.36 %	H 6.66 %	N 8.54 %
C <sub>9</sub> H <sub>11</sub> O <sub>2</sub> N requires.....	C 65.41 %	H 6.72 %	N 8.49 %

### dl-Threonine

*dl*-Threonine was synthesized from crotonic acid in accordance with the following reaction scheme, proposed by CARTER and WEST (1937). In order to separate *dl*-threonine from *dl*-allothreonine, the corresponding methoxy derivate was converted into formyl-*dl*-O-methylthreonine and formyl-*dl*-O-methylallothreonine, which, owing to their difference in solubility, were easily separated.







I—IV. *α-Bromo-β-methoxy-n-butyric acid* (IV) was synthesized according to the technique described by CARTER and WEST (1940). Crotonic acid and mercuric acetate were condensed in methyl alcohol into a not exactly identified addition product insoluble in methyl alcohol. By treating this addition product (II) with potassium bromide in an aqueous solution, we got potassium *α*-bromomercuri-*β*-methoxy-*n*-butyrate, which was brominated. The free acid, *α*-bromo-*β*-methoxy-*n*-butyric acid, was extracted with ether after acidification with hydrobromic acid. When the ether had been driven off, *α*-bromo-*β*-methoxybutyric acid was obtained as a viscous oil, which was distilled in a vacuum at 125—128° C. / 10 mm. Yield 74—87 %.

IV—VI. *Formyl-dl-O-methylthreonine* (VI). By treating *α*-bromo-*β*-methoxy-*n*-butyric acid with concentrated ammonia in an autoclave at 90—100° C., a mixture of dl-O-methylthreonine and dl-O-methylallothreonine was obtained. In order to separate these two substances, they were converted into the corresponding formyl derivatives (CARTER and WEST, *loc. cit.*) by treatment with formic acid in acetic acid anhydride. As the formyl derivative of dl-O-methylthreonine is less soluble than the corresponding derivate of dl-O-methylallothreonine, the result was that, on crystallization from a saturated solution, a product consisting mostly of formyl-dl-O-methylthreonine was obtained. On further crystallization, the resulting product consisted of pure formyl-dl-O-methylthreonine; m. p. 174—176° C. Yield 18—24 %.

VI—VIII. *dl-Threonine* (VIII). The formyl-dl-O-methylthreonine was hydrolyzed by heating with constant-boiling hydrobromic acid and, after evaporation, dl-threonine hydrobromide was obtained. The latter was dissolved in absolute alcohol and neutralized with ammonia; the dl-threonine thus precipitated was filtered off and dissolved in water and was crystallized after the addition of alcohol. The synthesis was carried out according to the method of CARTER and WEST (*loc. cit.*). Yield 70—75 %; m. p. 234—235° C. (dec.).

The dl-threonine was once again recrystallized in the following way:—

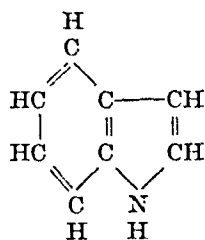
100 g dl-threonine was dissolved in 500 ml of warm distilled water and was boiled for half an hour with 5 g washed activated carbon. The solution was filtered in a warm condition and 3000 ml of warm absolute alcohol was added, whereupon the mixture was placed in the refrigerator over night. The precipitate was filtered off and washed with two portions of 200 ml absolute alcohol and 500 ml ether. Yield 90—98 g; m. p. 234—235° C. (dec.).

#### ANALYSIS.

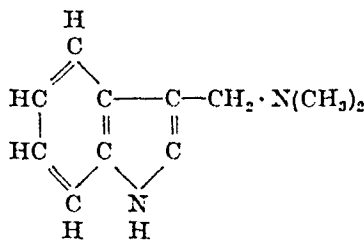
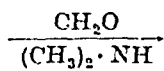
Found.....	C 39.91 %	H 7.63 %	N 11.54 %
C <sub>4</sub> H <sub>9</sub> O <sub>3</sub> N requires.....	C 40.31 %	H 7.62 %	N 11.74 %

## dl-Tryptophane

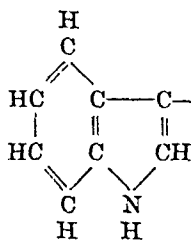
The production of dl-tryptophane was carried out in accordance with the following reaction scheme, proposed by SNYDER and SMITH (1944).



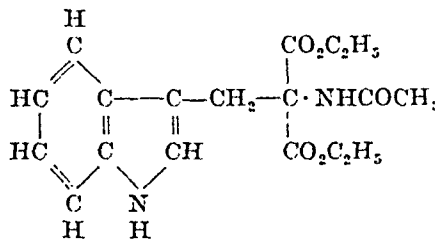
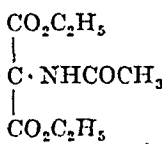
Indole (I)



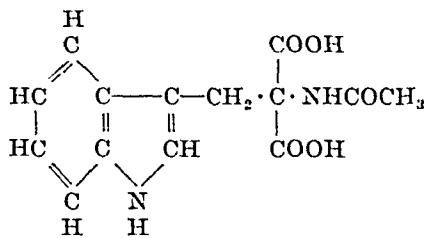
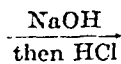
Gramine (3-Dimethylamino-methylindole) (II)



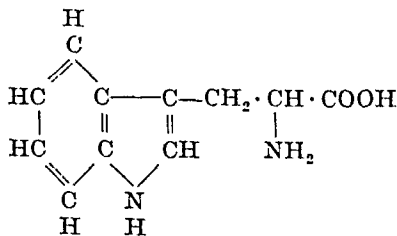
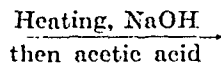
Gramine methiodide (III)



Ethyl α-acetamido-α-carbethoxy-β-(3-indole)-propionate (IV)



α-Acetamido-α-carboxy-β-(3-indole)-propionic acid (V)



dl-Tryptophane (VI)

I—III. *Gramine methiodide* (III). In accordance with the technique of SNYDER, SMITH and STEWARD (1944), gramine was produced from indole by treatment with dimethylamine and formaldehyde. The gramine thus obtained was caused to react with methyl iodide, so that gramine methiodide resulted. Yield 80—90 %.

III—IV. *Ethyl  $\alpha$ -acetamino- $\alpha$ -carbethoxy- $\beta$ -(3-indole)-propionate* (IV) was obtained by condensation of ethyl sodium acetaminomalonate and gramine methiodide in dioxane according to SNYDER and SMITH (*loc. cit.*). Ethyl acetaminomalonate was prepared according to the method described on p. 27. Yield of ethyl  $\alpha$ -acetamino- $\alpha$ -carbethoxy-(3-indole)-propionate 43—53 %; m. p. 156—158° C.

IV—V.  *$\alpha$ -Acetamino- $\alpha$ -carboxy- $\beta$ -(3-indole)-propionic acid* (V) was prepared by saponification of ethyl  $\alpha$ -acetamino- $\alpha$ -carbethoxy- $\beta$ -(3-indole)-propionate with sodium hydroxide; after acidification, the free acid (V) was obtained according to the method of SNYDER and SMITH (*loc. cit.*). The acid thus produced (V) contained some sodium chloride, but was nevertheless used in that condition for the continued synthesis. Yield 90—95 %; m. p. 136—139° C.

V—VI. *dl-Tryptophane*. From  $\alpha$ -acetamino- $\alpha$ -carboxy- $\beta$ -(3-indole)-propionic acid, dl-tryptophane was obtained by decarboxylation effected by boiling in water and hydrolysis by heating with sodium hydroxide. After treatment with washed activated carbon, the solution was acidified with acetic acid, to precipitate dl-tryptophane. The product thus obtained was dl-tryptophane with a melting point of 272—282° C. (*dec.*). The method was that proposed by SNYDER and SMITH (*loc. cit.*). Yield 68—69 %.

Further purification of the dl-tryptophane was carried out in the following way:—

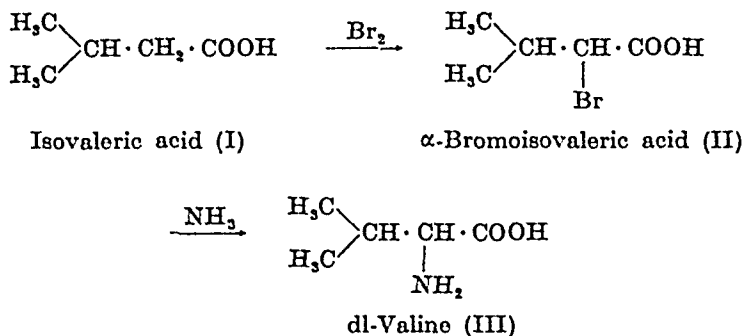
28 g dl-tryptophane was dissolved in 400 ml of distilled water, mixed with 10 g NaOH. The solution was filtered and 200 ml of 95 % alcohol was added. Whilst stirring, 15 ml of acetic acid was then added. The mixture was placed in the refrigerator over night; the precipitate was filtered off and washed with two portions of 80 ml cold distilled water, two portions of 80 ml absolute alcohol and two portions of 80 ml ether. The dl-tryptophane thus obtained, after drying in air, weighed 23—28 grams; m. p. 275—282° C. (*dec.*).

#### ANALYSIS.

Found.....	C 64.68 %	H 5.93 %	N 13.42 %
$C_{11}H_{12}O_2N_2$ requires.....	C 64.67 %	H 5.93 %	N 13.72 %

#### dl-Valine

This amino acid was synthesized according to a technique proposed by MARVEL (1940). The reaction scheme is shown below:—



I—II. *α-Bromoisovaleric acid* (II) was prepared from isovaleric acid, b. p. 176° C., by bromination in the presence of  $\text{PCl}_3$  according to the method of MARVEL (*loc. cit.*). The *α*-bromoisovaleric acid thus obtained was distilled in a vacuum at 110—125° C./15 mm. Yield 80—85 %.

II—III. *dl-Valine*. In accordance with the technique of MARVEL (*loc. cit.*) *dl*-valine was prepared by the treatment of the bromic acid with ammonia. The *dl*-valine was recrystallized in 50 % alcohol. Yield 30—35 %; m. p. 280—282° C. (dec.).

For further purification, the *dl*-valine was recrystallized in the following way:—

760 g *dl*-valine was dissolved in 6 litres of warm distilled water, and treated with 20 g washed activated carbon for half an hour. The mixture was filtered warm, and the filtrate was mixed with 6 litres of warm absolute alcohol. It was placed in the refrigerator over night. The precipitate was filtered off and washed with two portions of 1000 ml absolute alcohol and two portions of 1000 ml ether. When dried in air, the *dl*-valine weighed 540 grams; m. p. 280—282° C. (dec.).

#### ANALYSIS.

Found.....	C 51.22 %	H 9.41 %	N 11.93 %
$\text{C}_6\text{H}_{10}\text{O}_2\text{N}$ requires .....	C 51.24 %	H 9.47 %	N 11.96 %

#### Comments

In regard to the above-reported syntheses, certain comments seem to be called for:—

The above used methods for the production of *dl*-histidine (in accordance with the method of ALBERTSON and ARCHER, 1945), *dl*-isoleucine, *dl*-leucine, *dl*-lysine, *dl*-methionine, *dl*-phenylalanine, *dl*-threonine, *dl*-tryptophane and *dl*-valine were, broadly speaking, satisfactory and suitable for laboratory practice. With these methods the following amounts of amino acids were produced: *dl*-histidine 150 g, *dl*-isoleucine 900 g, *dl*-leucine 920 g,

dl-lysine HCl 650 g, dl-methionine 300 g, dl-phenylalanine 800 g, dl-threonine 380 g, dl-tryptophane 150 g and dl-valine 1300 g.

The synthesis of dl-arginine, on the other hand, was troublesome. The yields were particularly poor in the production of dl- $\delta$ -benzoylornithine from  $\delta$ -benzoylaminovaleric acid via  $\alpha$ -bromo- $\delta$ -benzoylaminovaleric acid.—From 4600 g adipic acid only 84 g dl-arginine HCl was obtained, which means a yield of 1.3 %!

The production of dl-histidine, as proposed by PYMAN (*loc. cit.*), from citric acid cannot be recommended. In the tests described above 15 g dl-histidine HCl, 2 H<sub>2</sub>O was obtained from 4000 g citric acid (yield 0.3 %!) after 3—4 months' work. The synthesis of dl-histidine is best carried out in accordance with the method of ALBERTSON and ARCHER (*loc. cit.*).

## CHAPTER V

### Experiments on Animals

The first part of the animal experiments related to the effect of the different amino acids essential for growth on the body weight and appetite when supplied in varying concentrations from 0 % until a toxic effect was observed. The essential feature of these experiments was the composition of a diet including all the amino acids essential for growth. For the reasons previously mentioned, the diet also contained dl-arginine, in order to obtain as rapid a growth as possible. From this diet one of the essential amino acids, of which the effect was to be investigated was excluded, and a series of diets with varying concentrations of the amino acid in question was arranged. Each diet was given to two animals. As a rule the following seven concentrations of the amino acid were tested: 0, 0.50, 1, 2, 4, 8, and 16 %. In certain cases where satisfactory growth was obtained with 0.5 %, tests were made also with 0.125 and 0.25 %, in order to obtain a more reliable estimate of the minimum concentration that would tend to maintain the body weight. Weight curves of the tests were made, and the change in weight as well as the intake of food during the 10 days of the experimental period was deter-

mined. On the basis of these results, graphs showing the relation between the changes in weight and the concentrations as well as between the intake of food and the concentration of the amino acid were drawn up.

As already mentioned, determinations of the changes in weight were made on two animals in regard to each concentration of the amino acid in the diet. The mean for changes in body weight at each concentration in an amino acid series was computed. The standard error of the mean ( $\epsilon_m$ ) in a series was obtained from the formula

$$\epsilon_m = \pm \sqrt{\frac{1}{4 \cdot n} \sum (W_1 - W_2)^2}$$

where  $n$  is the number of double determinations in the said amino acid series.  $W_1$  and  $W_2$  represent the changes in weight of two animals at the same concentration of the amino acid. The square deviations  $[(W_1 - W_2)^2]$  showed no definite correlation with the percentage of the respective amino acid in the diet. The means of the changes in weight at different concentrations of the various amino acids and the standard errors of the means in the different series are given in Table IX (p. 76).

The lowest concentration of the amino acid in question for maintenance of the body weight was determined on the graphs showing the connection between change in weight and the concentration of the amino acid under investigation.

For calculation of the concentrations which give the maximal growth, the author first attempted to express the observed figures for the increase in weight with the aid of exponential functions in order to obtain the concentrations mentioned. It was found, however, that the result was greatly dependent on the function selected. As the systematic variations in this material are small in comparison with the variations in weight changes, the author refrained from these complicated calculations. The concentrations for maximal growth are given with the assumption that a difference between two means of weight changes is significant if it exceeds twice the standard error of the mean.

All the concentrations above the highest required for maximal growth must be regarded as toxic, since in such cases the growth will fall according as the concentration of the amino acid

supplied is higher. In such a small material as this, it is difficult to determine this limit with any degree of accuracy. The concentration was therefore assumed to be toxic when there was a distinct and indubitably unfavourable effect on the growth. This has been considered to occur at the concentration which resulted in the body weight of the animals showing no change during the test or which caused the death of the animals in the course of the 10 days of the experimental period, in case it had been found that the next lower concentration tested would produce growth.

In each amino acid series the intake of food in default of the said amino acid is reported. The concentration of the respective amino acid when the appetite is best was determined as indicated above in regard to concentrations tending to promote maximal growth. The means of the intake of food at varying concentrations in the different amino acid series and the corresponding standard errors of the means are given in Table IX (p. 76). The calculations of these standard errors were made in the same manner as for those of changes in the body weight (page 45).

In order to obtain a basis of comparison, similar feeding tests were made with ordinary diet, a diet completely free from protein and amino acids, and a diet containing casein (15 %) in place of amino acids.

When some idea had been obtained as to the concentrations of the different essential amino acids required for maximum growth, protracted tests were made with a diet containing only the nine synthetic amino acids essential for normal growth.

It appeared from the feeding tests that the appetite deteriorated when an amino acid was missing from the diet. There was therefore reason to suspect that the decrease in weight was due to the poor appetite. In order to throw light on this matter, feeding tests were made for 10 days with a diet containing all the amino acids essential for growth, in optimal concentrations. Of this diet, the animals received per day an amount equivalent to that consumed on an average when the diet was devoid of an amino acid essential for growth.

As laboratory animals, white female rats with a weight of 43—51 grams were used. During the course of the test they were

kept in network cages of metal with one rat in each. The cages were placed in a room with a constant temperature of 22.5—23.5° C. Subject to the exception mentioned in the preceding paragraph, the animals were allowed to eat *ad libitum* of the experimental diets. They also had free access to water. The tests commenced at 9 a.m. and the rats as well as the food were weighed daily at that time.

Two days before each test, the rats were placed in their cages for pre-treatment. They then received a diet containing the same ingredients as the test diet besides an optimal amount of the essential amino acids. The object of this pre-treatment was to obviate the change in weight that may occur when animals are transferred from one diet to another. As a rule the rats lost 1—2 grams in weight after 24 hours, but then began to regain their weight on the second day.

### Preparation of the different diet mixtures

The amino acid-free part of the diet was composed of saccharose, cod liver oil, wheat-germ oil, salts, cellulose and crystalline vitamins. To this basic diet various mixtures of amino acids were added. The amino acid-free diet, per 100 grams of the complete diet, had the composition shown in the following Table IV (column 2).

This diet mixture is similar to that used by BORMAN, WOOD, BLACK, ANDERSON, OESTERLING, WOMACK, and ROSE (1946) in their investigations into the importance of l(+)-arginine for growth in rats. Their diet contained liver extract and biotin. In the tests recorded below, liver extract was excluded in view of its content of protein. For tests of this kind the biotin is not necessary.

The saccharose, cod liver oil and wheat-germ oil were the ordinary commercial products. The cellulose meal was produced by grinding filter paper in a ball-mill of porcelain. The salt mixture used was that specified as No. 12 by JONES and FOSTER (1942).

All the vitamins were crystalline products. The saccharose, cod liver oil, wheat-germ oil and the cellulose were found, on nitrogen analysis according to KJELDAHL with the technique described by PETERS and VAN SLYKE (1932), to contain quite small



TABLE IV. Composition of the amino acid-free part of the test diet.

1	2	3
	The amino acid-free diet contains per 100 g of the whole diet g	M <sub>1</sub> g
Saccharose .....	So much that the amino acid-free diet mixture, to- gether with the amino- acids, amounts to 100 g	560
Cellulose meal .....	2	20
Salt mixture .....	4	40
Cod liver oil .....	0.5	5
Wheat germ oil .....	1.5	15
Inositol .....	0.1	1
Choline chloride .....	0.2	2
Aneurine HCl .....	0.0005	0.005
Riboflavin .....	0.001	0.010
Pyridoxine HCl .....	0.0005	0.005
Nicotinamide .....	0.0005	0.005
Ca d-pantothenate .....	0.0025	0.025
p-Aminobenzoic acid .....	0.03	0.300
2-Methyl-1, 4-naphthoquinone .....	0.0002	0.002
Folic acid .....	0.001	0.010
Total		643.362 g

Column 2 shows the amount of the different constituents per 100 g of the whole diet. Column 3 shows the composition of the "basic diet" M<sub>1</sub>. The latter is given in the proportion of 96.5 to 150 g of the whole diet.

amounts of nitrogen. The amounts of nitrogen supplied with this part of the diet are shown in Table V.

TABLE V. Nitrogen percentage in amino acid-free constituents of the test diet.

	Nitrogen percentage in the constituents
Saccharose .....	0.020
Cellulose meal .....	0.025
Salt mixture .....	0.008
Cod liver oil .....	0.010
Wheat-germ oil .....	0.008

From Table V it can be estimated that the part of the test diets free from amino acids and vitamins contains between 0.012 and 0.019 % N. The variations are due to the amount of saccharose contained in the diet. At most 0.19 mg N per g of the test diet is supplied in this way. This nitrogen may be derived from protein or from amino acids. As indicated by the tests, it is scarcely likely that these minute amounts of nitrogen, even if they should consist of protein or amino acids, play any rôle in the results of the tests.

The test diets were prepared in the following way:—A nitrogen-free diet, which was termed  $M_1$ , with the composition described in Table IV (column 3), was first arranged. As a rule a diet amounting altogether to 150 grams was mixed, the required amount of the amino acid-free diet ( $M_1$ ) being 96.5 grams. For each experimental series, an amino acid mixture containing all the amino acids except the one to be tested was prepared. The composition of the different mixtures of amino acids is shown by the following Table VI. Sufficient sodium bicarbonate was included in the diet to neutralize the hydrochlorides present. In the first series with dl-lysine and dl-leucine (Table VI, columns 2 and 3) the proportions of the amino acids in the mixtures were the same as those used in tests recorded in a previous work (WRETLIND 1948), except that dl-arginine was supplied in a concentration of only 0.2 % of the diet, which, according to ROSE (1937), suffices for optimal growth. In the following series the concentration of dl-histidine, dl-isoleucine, dl-methionine, dl-phenylalanine, dl-threonine, dl-tryptophane and dl-valine was changed to the nearest test concentration (0.5, 1, 2 or 4 %). Later on during the progress of the investigations, the concentrations in succeeding series were modified in such a way that the amino acids were supplied in concentrations which, as shown by preceding series, had been found to produce maximal growth.

The amino acid mixtures were finely pulverized in a so-called Turmix apparatus, which operates with rapidly rotating knives, so that a flour-like powder is obtained. The estimated amount of this amino acid mixture (see the last row of Table VI) besides the requisite amount of the amino acid to be tested was then mixed with 96.5 grams of the amino acid-free diet  $M_1$  as well as

TABLE VI. Composition of the different

1	2		3		4		5		6	
	Composition of the amino acid mixtures for test									
	dl-Lysine		dl-Leucine		dl-Valine		dl-Threonine		dl-Isoleucine	
	g	%	g	%	g	%	g	%	g	%
dl-Histidine.....	10.4	0.69	10.4	0.69	7.5	0.50	7.5	0.50	7.50	0.50
dl-Isoleucine.....	43.0	2.87	43.0	2.87	30.0	2.00	30.0	2.00		
dl-Leucine.....	43.0	2.87			30.0	2.00	30.0	2.00	30.0	2.00
dl-Lysine HCl.....			37.4	2.00	37.4	2.00	37.4	2.00	37.4	2.00
dl-Methionine.....	21.6	1.44	21.6	1.44	15.0	1.00	15.0	1.00	15.0	1.00
dl-Phenylalanine ...	17.3	1.15	17.3	1.15	15.0	1.00	15.0	1.00	15.0	1.00
dl-Threonine.....	34.5	2.30	34.5	2.30	30.0	2.00			30.0	2.00
dl-Tryptophane ....	8.6	0.57	8.6	0.57	7.5	0.50	7.5	0.50	7.5	0.50
dl-Valine.....	60.5	4.00	60.5	4.00			60.0	4.00	45.0	3.00
dl-Arginine HCl....	3.7	0.20	3.7	0.20	3.7	0.20	3.7	0.20	3.7	0.20
NaHCO <sub>3</sub> .....	1.5		18.5		18.5		18.5		18.5	
Total	244.1		255.5		194.6		224.6		209.6	
Amounts of the various amino acid mixtures required to make 150 grams diet	24.41		25.55		19.46		22.46		20.96	

The table shows 1) the weight in grams of the amino acids in the mixtures and 2) the intended percentage concentration of the different amino acids in the diet. The amount of the different mixtures required to make up 150 grams of the total diet is given in the bottom row. The percentages of dl-arginine HCl and dl-lysine HCl are estimated according to the free bases.

sufficient saccharose to bring the final weight up to 150 grams. In this way diet series were arranged with the said amino acid in previously mentioned concentrations.

The mixtures for the tests with the nine amino acids essential for growth, singly or with the addition of dl-arginine, are shown in Table VI (columns 11 and 12, respectively), which also indicates the amount of those mixtures required to make up the final diet; the latter includes 96.5 grams of the amino acid-free diet M<sub>1</sub> and sufficient saccharose to bring the total weight up to 150 grams.

The pre-treatment diet was obtained by mixing 22.9 grams of

amino acid mixtures used in the feeding tests.

7		8		9		10		11		12		13	
showing the effect of varying concentrations of:								Mixture for test with amino acids essential for growth		Mixture for test with amino acids essential for growth plus dl-arginine		Amino acids for pre-treatment diet	
dl-Methionine		dl-Phenylalanine		dl-Tryptophane		dl-Histidine							
g	%	g	%	g	%	g	%	g	%	g	%	g	%
7.5	0.50	7.5	0.50	7.5	0.50			7.5	0.50	7.5	0.50	5.0	0.5
30.0	2.00	30.0	2.00	30.0	2.00	60.0	4.00	60.0	4.00	60.0	4.00	20.0	2.0
30.0	2.00	30.0	2.00	30.0	2.00	30.0	2.00	30.0	2.00	30.0	2.00	20.0	2.0
37.4	2.00	37.4	2.00	37.4	2.00	37.4	2.00	37.4	2.00	37.4	2.00	25.0	2.0
		15.0	1.00	11.3	0.75	11.3	0.75	11.3	0.75	11.3	0.75	10.0	1.0
15.0	1.00			15.0	1.00	15.0	1.00	30.0	2.00	30.0	2.00	10.0	1.0
15.0	1.00	15.0	1.00	15.0	1.00	15.0	1.00	15.0	1.00	15.0	1.00	10.0	1.0
7.5	0.50	7.5	0.50			5.7	0.38	5.7	0.38	5.7	0.38	5.0	0.5
45.0	3.00	45.0	3.00	45.0	3.00	45.0	3.00	45.0	3.00	45.0	3.00	30.0	3.0
3.7	0.20	3.7	0.20	3.7	0.20	3.7	0.20			3.7	0.20	2.5	0.2
18.5		18.5		18.5		18.5		17.0		18.5		15.0	
209.6		209.6		213.4		241.6		258.9		264.1		152.5	
20.96		20.96		21.34		24.16		25.89		26.41		22.9	

the amino acid mixture for that diet (col. 13) with 96.5 grams of  $M_1$  and 30.6 grams of saccharose.

The mixture of the diets was made in a so-called "Elektrolux household assistant" (an apparatus for automatic grinding, mixing etc.) and took one hour.

The different feeding tests were made in the order of description.

### Feeding tests and their results

#### dl-Lysine

In the tests, the following concentrations of dl-lysine (the free base) were used: 0, 0.25, 0.50, 1, 2, 4, 8 and 16 %.

The weight curves are reproduced in Figs. 1 and 2. The connection between the change in weight and the concentration of dl-lysine is seen in Fig. 3, whilst the relation between the intake of food and the concentrations is indicated by Fig. 4.

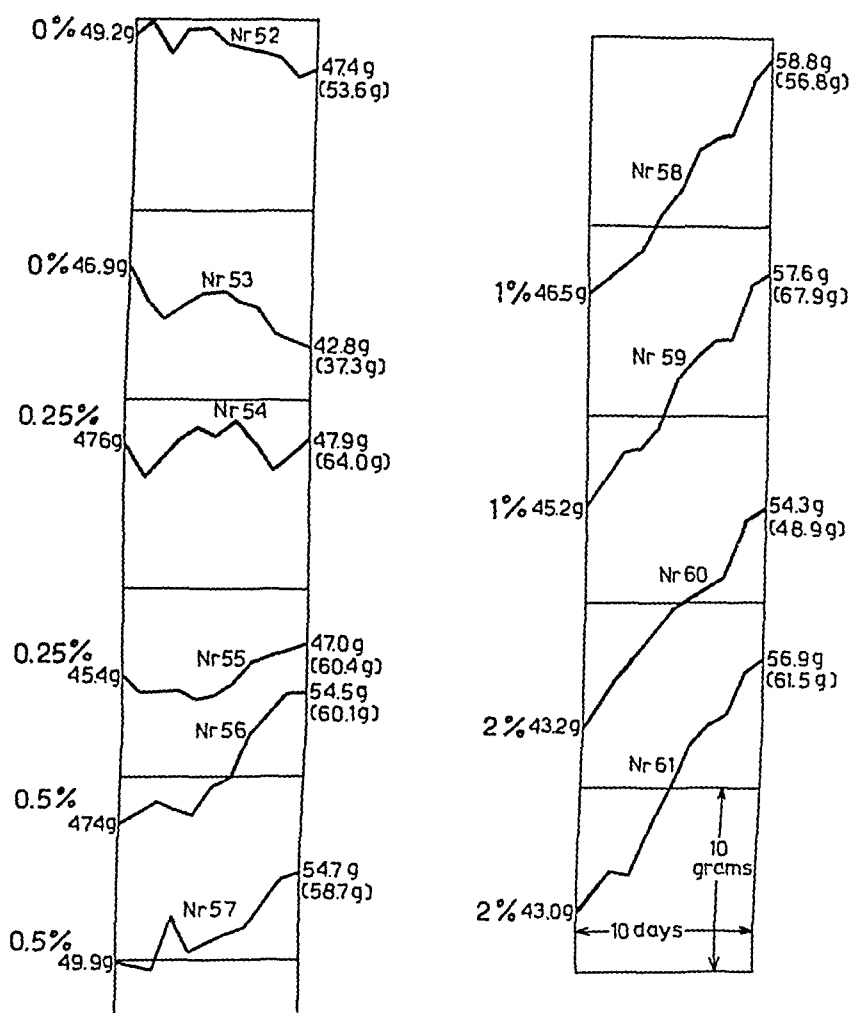


Fig. 1. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-lysine. — The abscissa indicates the number of days and the ordinate the body weight. The concentration of the dl-lysine in the diet is shown in front of the curves. At the beginning and end of each curve the initial and final weights, respectively, are indicated. The figure in brackets after the curve denotes the amount of food eaten by the rat during the experimental period.

**RESULT.** The animals that had received the lysine-free diet showed no other symptoms than a decline in weight: they were lively during the whole course of the test. One of the rats who had received 8 % dl-lysine showed excoriations and minor haemorrhages round the anus and on the median side of the tarsi of the forefeet. At a dl-lysine concentration of 16 % two out of three rats died on the 8th and 9th day, respectively (weight

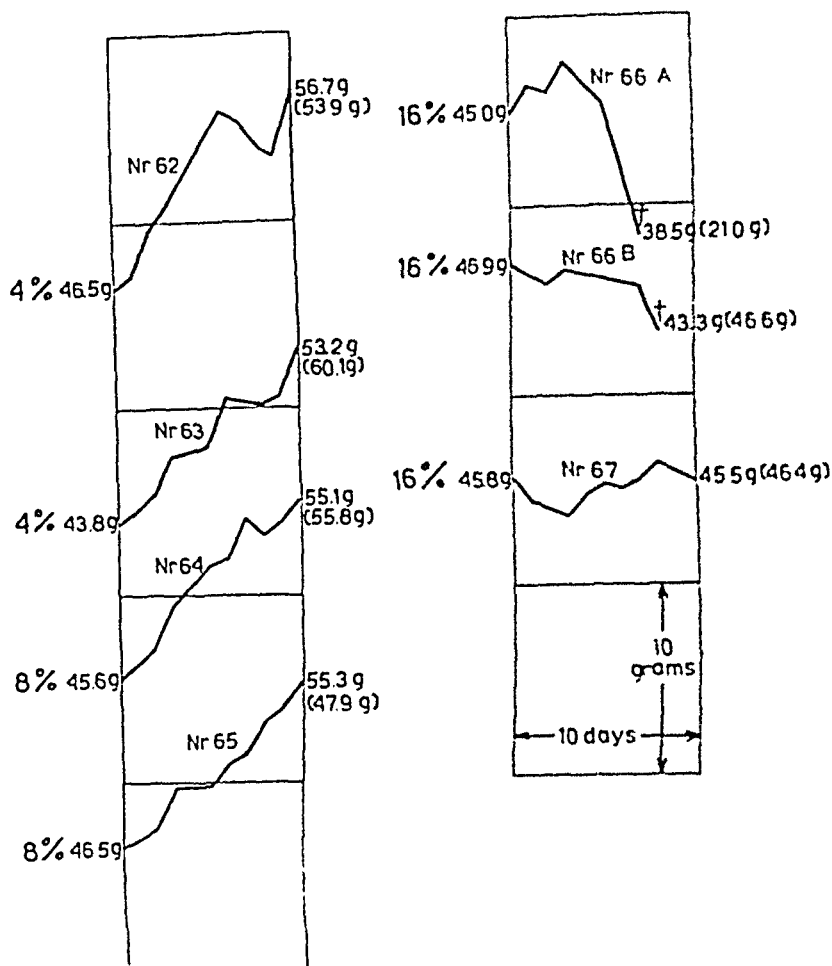


Fig. 2. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-lysine. — Notation the same as in Fig. 1.

curves 66 A and 66 B, Fig. 2). Both these rats showed sores and petechiae at the root of the tail and round the anus.

As may be seen from the weight curves, the fall in weight in default of lysine amounted to  $3.0 \pm 0.9$  grams per 10 days. At a concentration of ca. 0.2% dl-lysine no change in the body weight appeared (Fig. 3), maximal growth being obtained at a concentration of 1—2%. A distinct toxic effect resulted, as already mentioned, at 16%.

In default of dl-lysine in the diet the appetite deteriorated ( $46 \pm 4.9$  g/10 days), whereas it improved when this amino acid was supplied (Fig. 4), being maximal at a concentration of 0.25—4%; at higher concentrations some loss of appetite resulted.

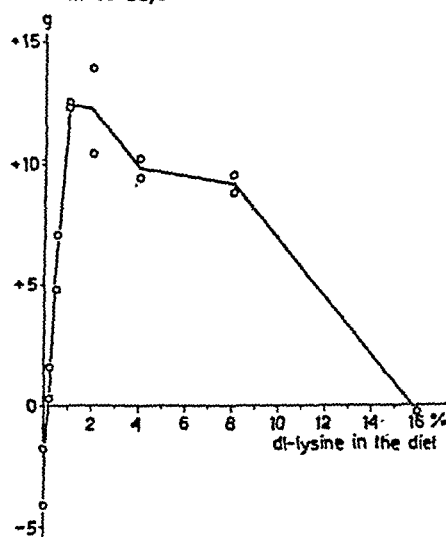
Change of body weight  
in 10 days

Fig. 3.

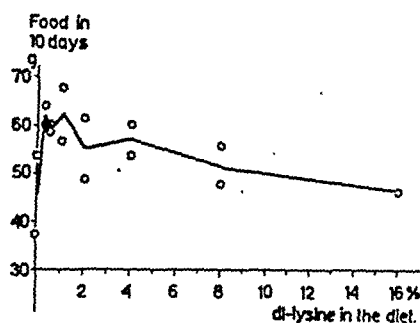


Fig. 4.

Fig. 3. Changes in weight during the experimental period in relation to the concentration of dl-lysine in the diet. — The ordinate shows the changes, in grams per experimental period (10 days), and the abscissa the percentage of dl-lysine in the diet. Each circle indicates a test on one animal. The averages of the tests with the different concentrations are connected with one another by the curve.

Fig. 4. Connection between the intake of food and the concentration of dl-lysine in the diet. — The ordinate indicates, in grams, the intake of food during 10 days, and the abscissa the percentage of dl-lysine in the diet. Each test is represented by a small circle on the curve. The averages of the tests are connected by the curve.

### dl-Leucine

The tested concentrations of dl-leucine were 0, 0.5, 1, 2, 4, 8, and 16 %. The weight curves and the connection between change in weight and concentration, as well as the connection between intake of food and concentration, are shown in Figs. 5, 6, and 7, respectively.

RESULT. Out of three rats on a leucine-free diet, one died on the 9th day. During the preceding days it was apathetic and dirty, but otherwise showed no external symptoms (weight curve No. 69 B, Fig. 5). The two surviving rats were both apathetic and dirty after 10 days, but after a further one or two days recovered on ordinary diet.

Figs. 5 and 6 indicate that in default of dl-leucine the animals

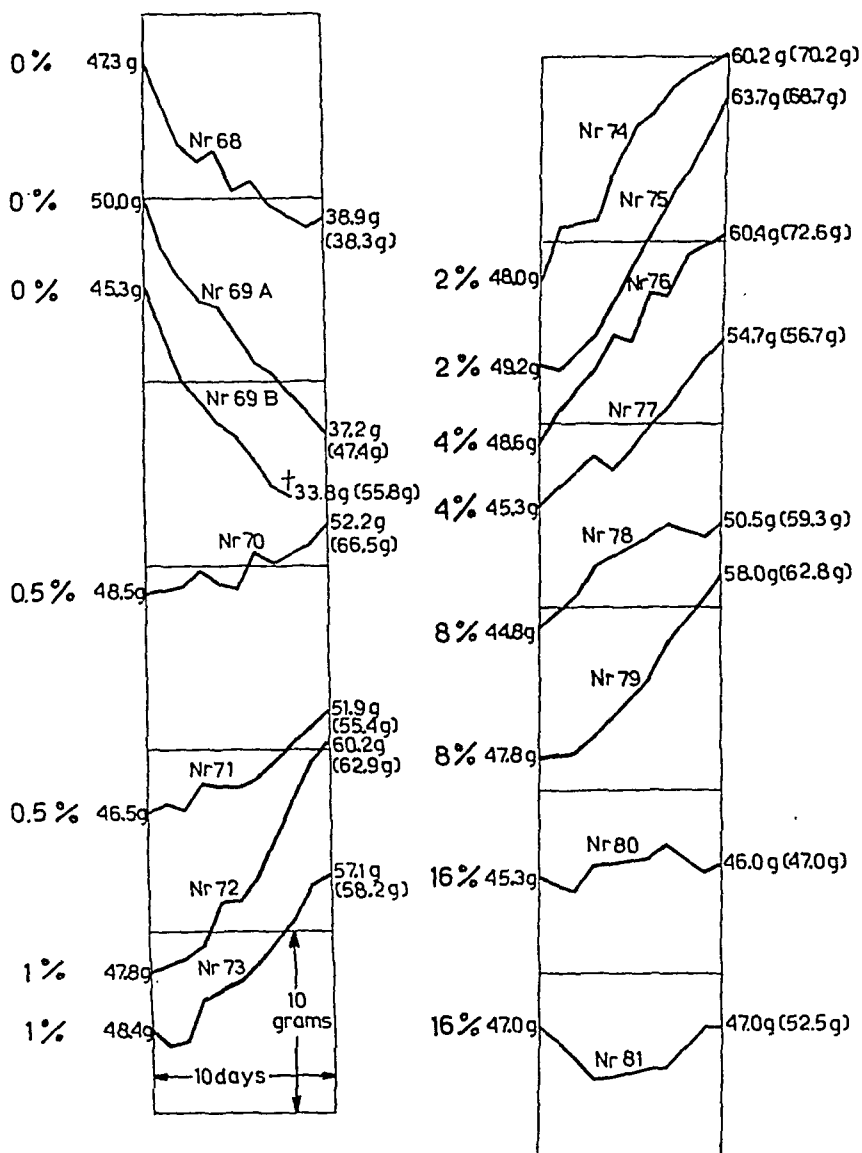


Fig. 5. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-leucine. — The concentrations of the dl-leucine are shown in front of the curves. Other notation the same as in Fig. 1.

lost  $10.6 \pm 1.6$  grams per 10 days. At a concentration of ca. 0.4 % dl-leucine no change in the body weight appeared, maximal growth being produced when the concentration of dl-leucine in the diet amounted to 1—4 %. If the concentration was raised to 16 %, the growth in weight completely ceased (toxic effect).



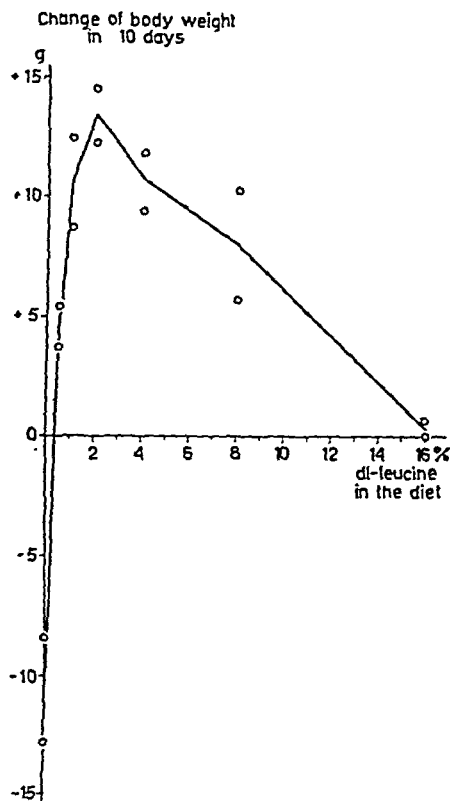


Fig. 6.

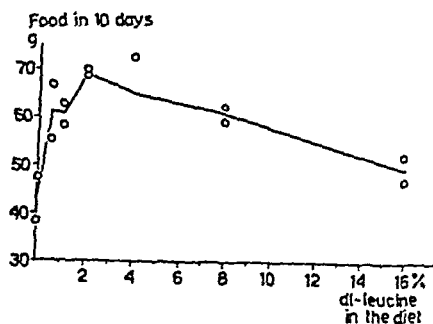


Fig. 7.

Fig. 6. Changes in weight during the experimental period (10 days) in relation to the concentration of dl-leucine in the diet.—Notation the same as in Fig. 3.

Fig. 7. Connection between the intake of food and the concentration of dl-leucine in the diet.—Notation the same as in Fig. 4.

As shown in Fig. 7, it may be observed that in default of dl-leucine the intake of food during the 10 days of the test was reduced to  $43 \pm 4.3$  grams. When dl-leucine was added, the appetite improved, being maximal at 0.5—8 % of the said amino acid in the diet. At the concentration of 16 %, however, the intake of food was again diminished.

### dl-Valine

The tested concentrations of dl-valine were 0, 0.5, 1, 2, 4, 8, and 16 %. The weight curves and the connections between change in weight or appetite and the concentrations are shown in Figs. 8, 9, and 10.

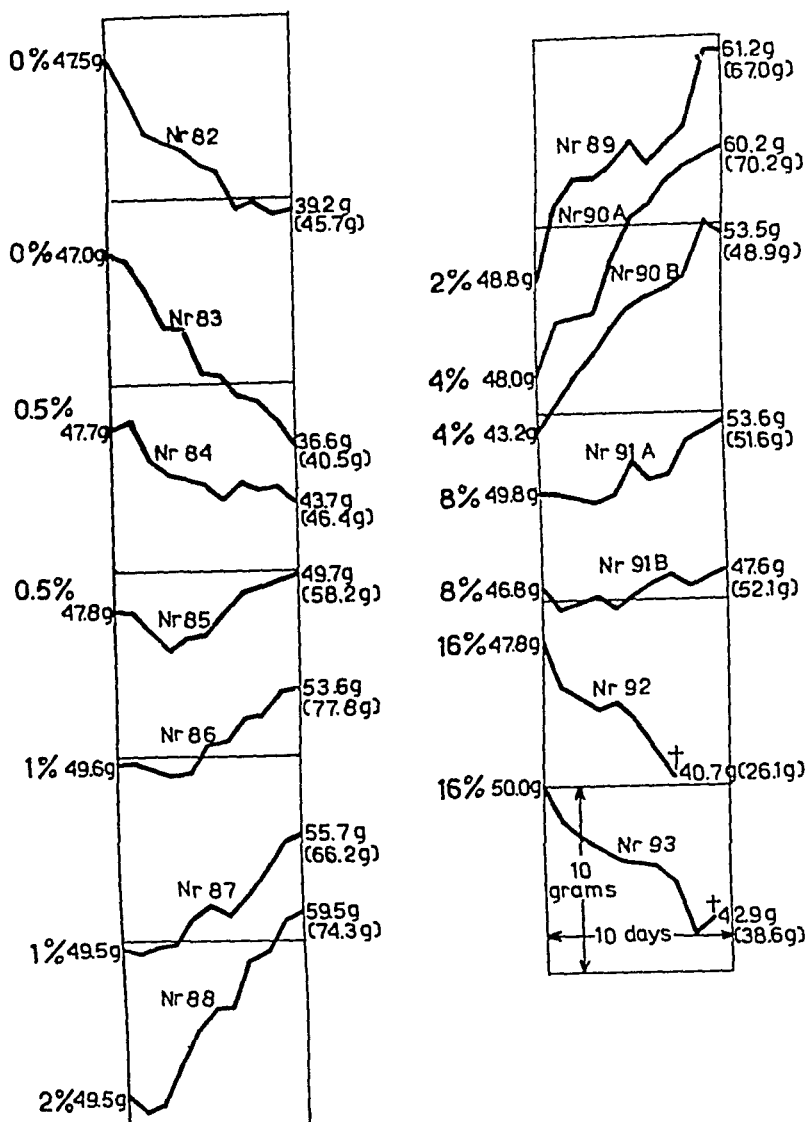


Fig. 8. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-valine.—The concentration of the dl-valine is shown in front of each curve. Notation the same as in Fig. 1.

RESULT. In none of the tests with a 0—8 % concentration of dl-valine were any noteworthy external symptoms observed, apart from the changes in weight. The two rats fed on the test diet containing 16 % valine died, respectively, on the 8th and 10th day (weight curves Nos. 92 and 93, Fig. 8). The rats were dirty

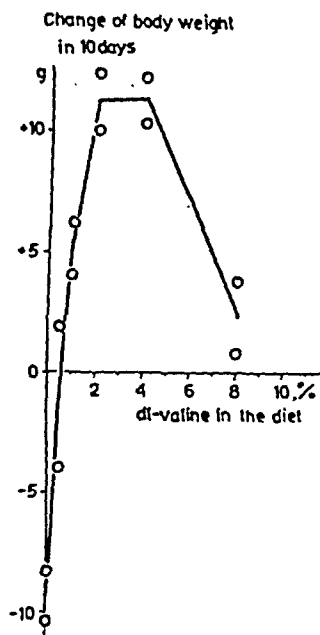


Fig. 9.

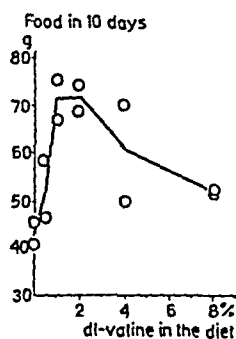


Fig. 10.

Fig. 9. Changes in weight in rats during 10 days, in relation to the concentration of dl-valine in the diet. — Notation the same as in Fig. 3.

Fig. 10. Connection between the intake of food and the concentration of dl-valine in the diet. — Notation the same as in Fig. 4.

and during the last few days of the test had slight diarrhea, but otherwise showed no external symptoms.

As may be seen from Fig. 8, the animals lost markedly in weight in default of dl-valine, the decrease being  $9.4 \pm 1.6$  grams per 10 days. At a concentration of ca. 0.6% dl-valine in the diet the animals showed no change in weight; for maximal growth 2—4 % dl-valine in the diet was required (Fig. 11). On a diet containing 8 % dl-valine a poor growth was obtained, and at 16 % the dl-valine, as stated above, had a toxic effect.

From Fig. 10 it is evident that in default of dl-valine the intake of food was rather small, being merely about  $43 \pm 5.8$  grams per 10 days. When the said amino acid was supplied the intake began to increase, being maximal at a concentration of 1—2 % dl-valine in the diet, but again diminishing at higher concentrations.

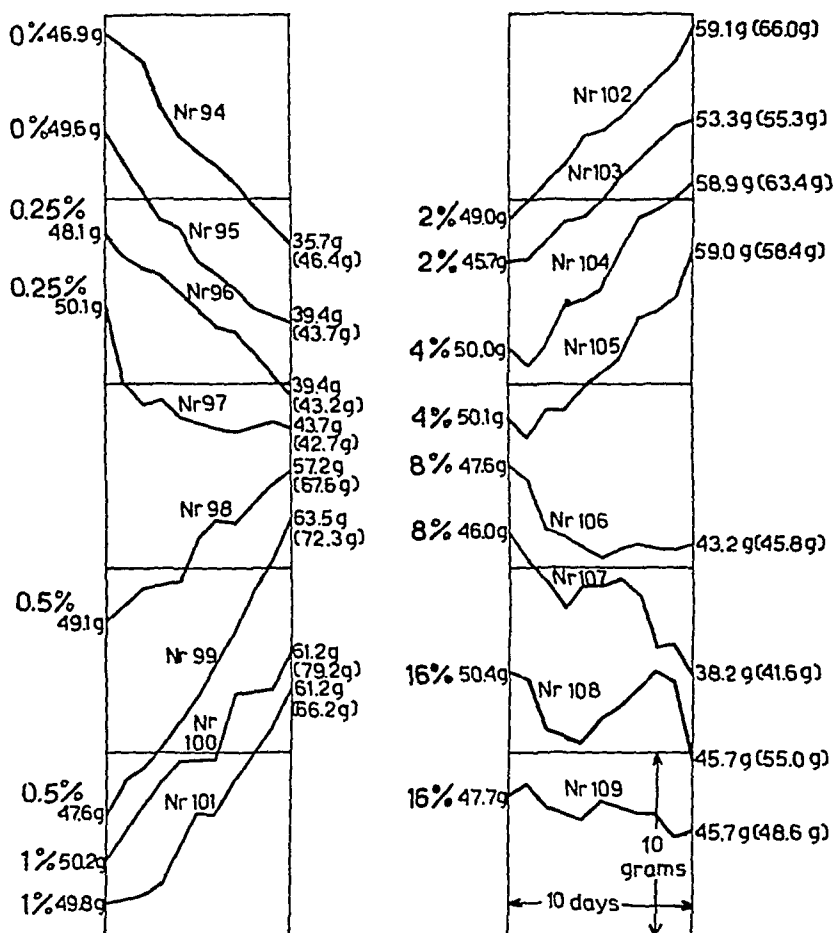


Fig. 11. Curves showing the growth of rats on a diet containing 9 amino acids and different concentrations of dl-threonine. — The concentrations of the dl-threonine are shown in front of the curves. Notation the same as in Fig. 1.

### dl-Threonine

The concentrations of dl-threonine used for the tests were 0, 0.25, 0.5, 1, 2, 4, 8, and 16 %.

The weight curves obtained with these concentrations are shown in Fig. 11. The connection between the changes in weight and the concentrations of dl-threonine are shown in Fig. 12, whilst the correlation between the concentration and the intake of food is seen from Fig. 13.

**RESULT.** The two rats fed on a diet with 16 % dl-threonine had diarrhea during the last few days of the 10 days' test. Apart

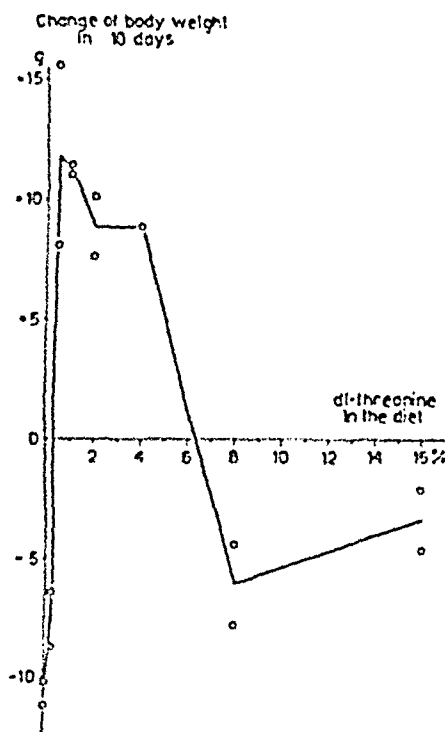


Fig. 12.

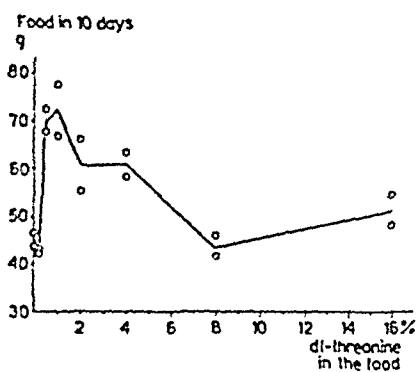


Fig. 13.

Fig. 12. Changes in weight in rats during 10 days in relation to the concentration of dl-threonine in the diet.—Notation the same as in Fig. 3.

Fig. 13. Connection between the intake of food and the concentration of dl-threonine in the diet.—Notation the same as in Fig. 4.

from the changes in weight, no other noteworthy external symptoms were observed.

Fig. 11 shows that with a lack of dl-threonine in the diet the body-weight of the animals diminished by  $10.7 \pm 1.7$  grams in ten days. At a concentration of 0.4 % dl-threonine the weight was just maintained, maximal growth being obtained at 0.5—4 % (Fig. 12). If the concentration of dl-threonine was raised, the increase in weight was retarded, so that at 6—7 % it had completely ceased (toxic effect). The concentrations of 8 and 16 % in the diet led to a decrease in weight (Fig. 12).

Fig. 13 shows that the intake of food was dependent on the concentration of dl-threonine in the diet. In the complete absence of this amino acid the intake of food was only about  $45 \pm 3.5$  grams in 10 days, but increased if dl-threonine was added to the diet. The appetite was maximal at 0.5—1 per cent. At higher concentrations the intake of food again diminished.

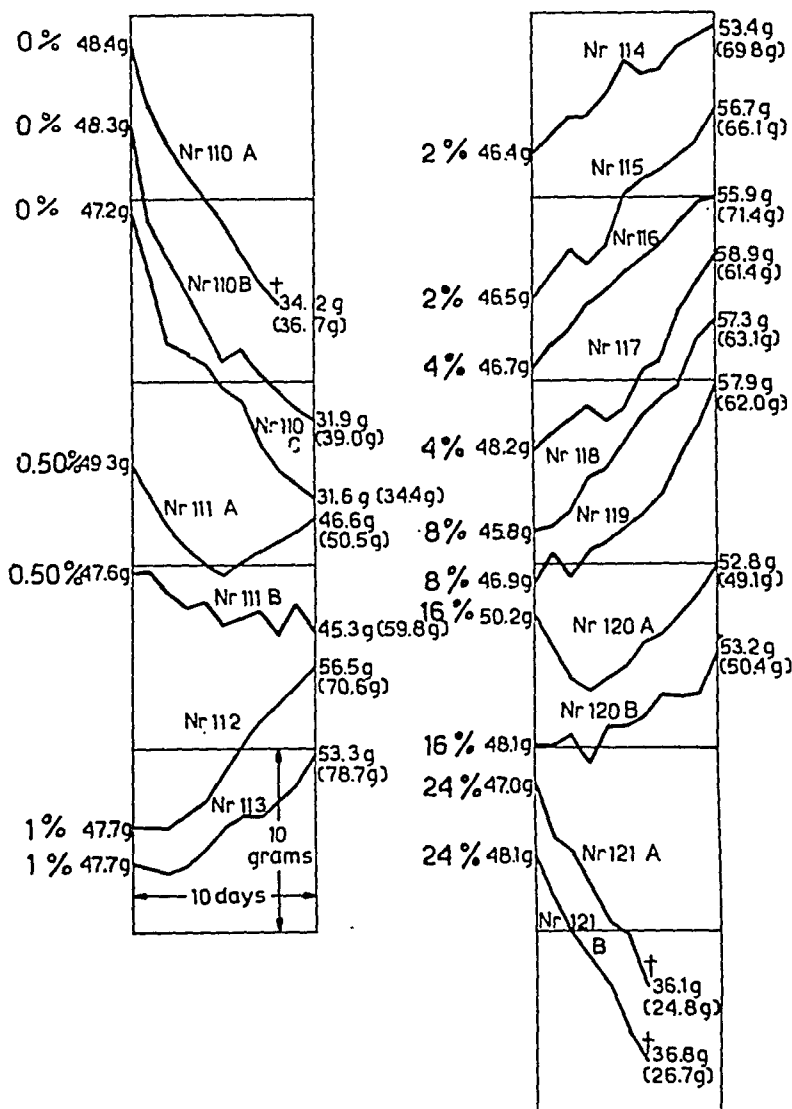


Fig. 14. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-isoleucine. — The concentration of the dl-isoleucine in the diet is shown in front of each curve.

### dl-Isoleucine

Tests were made with the following concentrations of dl-isoleucine: 0, 0.5, 1, 2, 4, 8, 16, and 24 %.

The weight curves obtained in these tests are shown in Fig. 14.

The changes in weight and variations in appetite according to the concentrations of dl-isoleucine are shown still more distinctly by Figs. 15 and 16.

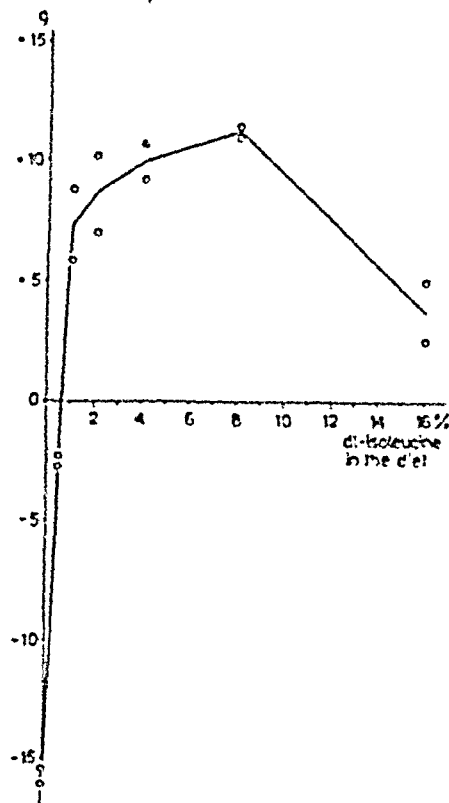
Change of body weight  
in 10 days

Fig. 15.

Food in 10 days

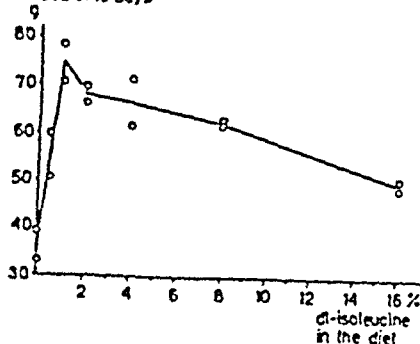


Fig. 16.

Fig. 15. Changes in weight in rats during the course of 10 days in relation to the concentration of dl-isoleucine in the diet. — Notation the same as in Fig. 3.

Fig. 16. Connection between the intake of food and the concentration of dl-isoleucine in the diet. — Notation the same as in Fig. 4.

**RESULT.** One of the three rats fed on a diet without isoleucine died on the 9th day (weight curve No. 110 A, Fig. 14). During the last few days of the test it was very emaciated and dirty, but otherwise showed no external symptoms. The two others markedly declined in weight, were dirty and emaciated during the last few days of the test. When they were put on ordinary diet, one of them after some days was again clean and increased in weight, whereas the other died on the 2nd day after the end of the experimental period.

In the other tests with dl-isoleucine in concentrations up to 16 % inclusive, no noteworthy symptoms were observed, apart

from the changes in weight. When the animals received a diet with 24 % dl-isoleucine, they were affected on the 3rd day with diarrhea, could not keep themselves clean, were apathetic and died at the end of the 7th day. Apart from the diarrhea, the state of emaciation and the uncleanness, no external symptoms were observed. In the course of the first 6 days (weight curves No. 121 A and B, Fig. 14) the rats had lost 11.3 and 10.9 grams in weight, respectively.

At 0 % isoleucine the result was a loss in weight of  $16.0 \pm 1.0$  grams per 10 days (Fig. 14). The curve in Fig. 15 shows that at a concentration of ca. 0.6 % dl-isoleucine the animal's original weight was just maintained. The optimal concentration may be estimated to lie between 4 and 8 per cent. Should the concentration of dl-isoleucine be raised considerably higher, viz. up to 24 %, the result, as already mentioned, was a distinctly toxic effect.

From Fig. 16 it appears that in default of isoleucine the appetite was poor, the consumption amounting merely to  $37 \pm 3.2$  grams per 10 days; that when this amino acid was supplied in quite a small amount the appetite began to improve, the consumption during the stated period reaching its maximum at a concentration of 1 %; and that if the concentration of the acid was raised any further, the appetite tended to diminish.

### dl-Methionine

The concentrations tested were 0, 0.125, 0.25, 0.5, 1, 2, 4, and 8 %. The resulting weight curves are shown in Fig. 17. On the basis of these values, the connection between the concentration of dl-methionine and the changes in weight or consumption of food are illustrated by the curves in Figs. 18 and 19, respectively.

RESULT One of the three rats fed on the methionine-free diet died on the 10th day (weight curve No. 122 A, Fig. 17) without any noteworthy external symptoms. One of those fed on the diet with 4 % dl-methionine died on the day after the termination of the experimental period (weight curve No. 134, Fig. 17). In this case bleeding from the nose was observed on the 10th day, but otherwise no noteworthy external symptoms. In the other rats no other symptoms than the changes in weight were observed.

As may be seen from Fig. 17, the result of the methionine-free



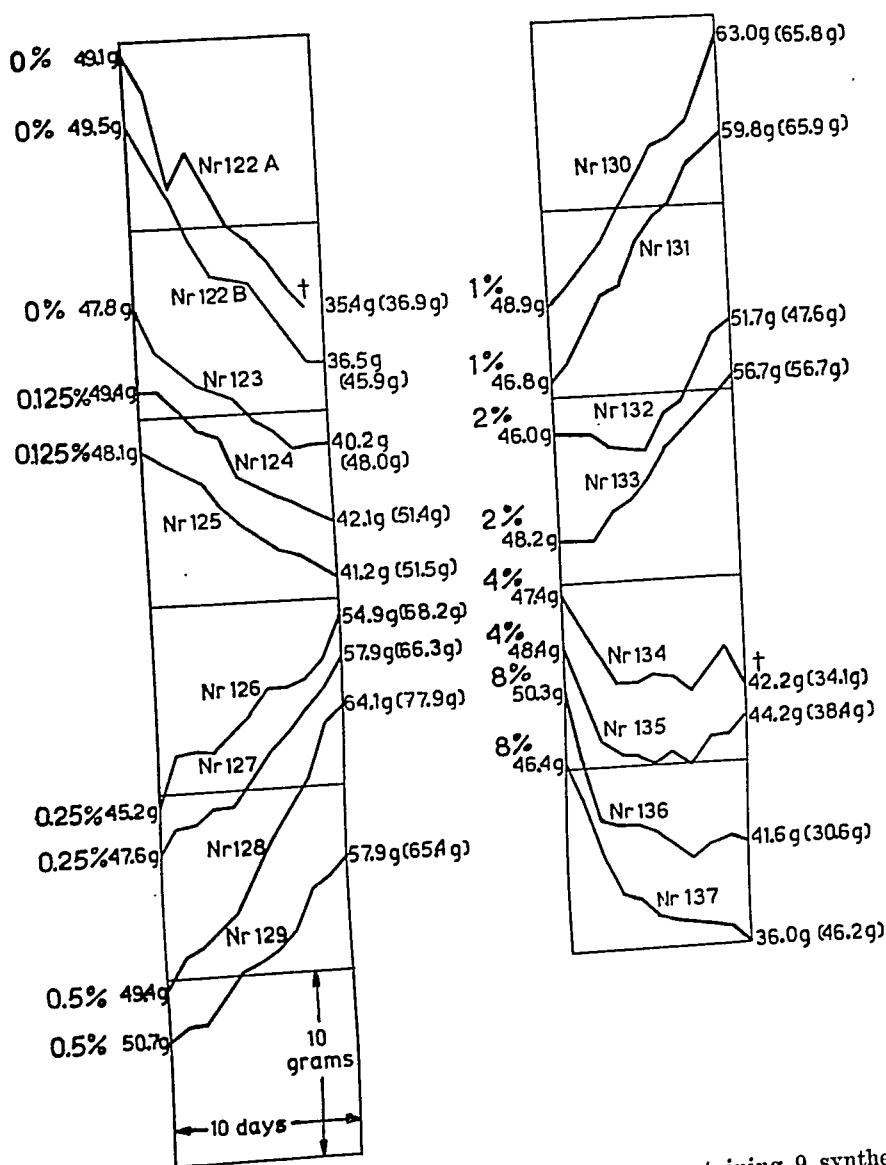


Fig. 17. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-methionine. — The concentrations of the dl-methionine in the diets are shown in front of the curves. Notation the same as in Fig. 1.

diet was a fall in weight of  $10.3 \pm 1.8$  grams per 10 days. At ca. 0.2 % dl-methionine in the diet the weight was just maintained, optimal growth being obtained at a concentration of 0.25—1 % (Fig. 18). When the concentration was increased, a poorer rise in weight resulted, so that at about 3 % it again showed complete

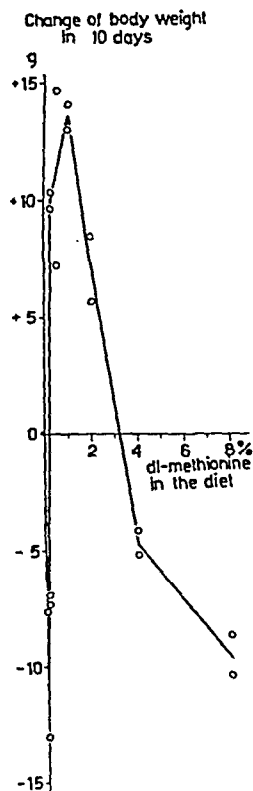


Fig. 18.

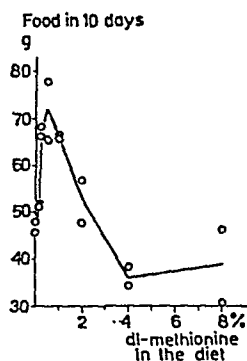


Fig. 19.

Fig. 18. Changes of weight in rats during the course of 10 days in relation to the concentration of dl-methionine in the diet.—Notation the same as in Fig. 3.

Fig. 19. Connection between the consumption of food and the concentration of dl-methionine in the diet.—Notation the same as in Fig. 4.

cessation. Concentrations of 4 and 8 % dl-methionine in the diet had a distinctly toxic effect, resulting in a marked fall of the weight by  $4.7 \pm 1.8$  grams and  $9.6 \pm 1.8$  grams, respectively.

From Fig. 19 it is evident that with a lack of methionine in the diet the appetite was poor, the consumption being only about  $47 \pm 4.0$  grams in 10 days. A marked improvement was shown on the addition of dl-methionine. At 0.25—1 % dl-methionine in the diet the consumption of food reached its maximum. If the concentration was increased, the appetite deteriorated, so that at 4 and 8 % it was poorer than on the methionine-free diet.

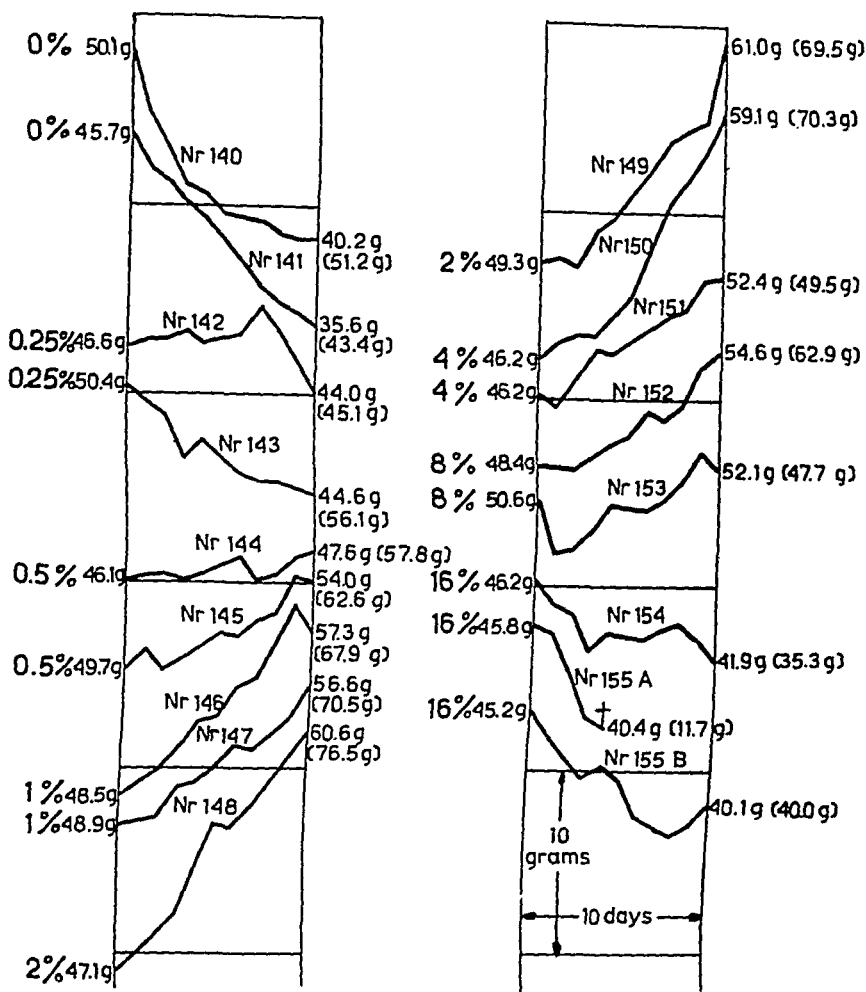


Fig. 20. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-phenylalanine.—The concentrations of dl-phenylalanine in the diet are shown in front of the curves. Notation the same as in Fig. 1.

### dl-Phenylalanine

In the experiments, diets containing the following concentrations of dl-phenylalanine were tested: 0, 0.25, 0.5, 1, 2, 4, 8, and 16 %.

The weight curves thus obtained are shown in Fig. 20. From Fig. 21 it is seen how the weight changed in the course of 10 days on modification of the concentration. The connection between the concentration of dl-phenylalanine and the appetite is indicated in Fig. 22.

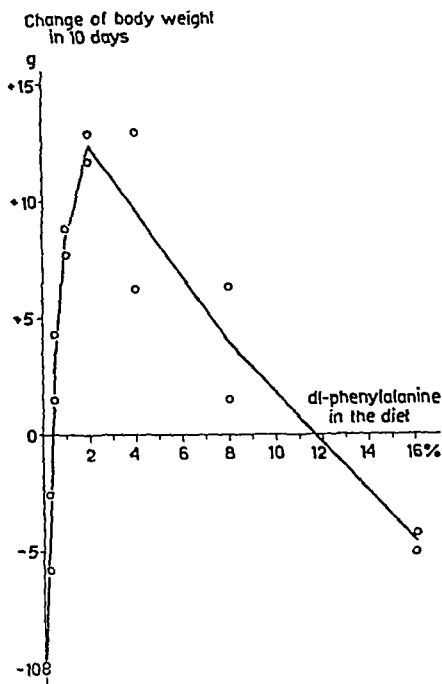


Fig. 21.

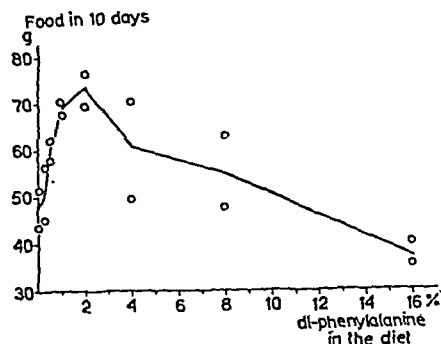


Fig. 22.

Fig. 21. Changes in weight in rats during the course of 10 days in relation to the concentration of dl-phenylalanine in the diet. — Notation the same as in Fig. 3.

Fig. 22. Connection between the consumption of food and the concentration of dl-phenylalanine in the diet. — Notation the same as in Fig. 4.

**RESULT.** One of the three rats fed on a diet containing 16 % dl-phenylalanine died on the 5th day after a decline in weight of 5.4 grams in 4 days (weight curve No. 155 A, Fig. 20). No external symptoms were observed and on macroscopic examination of the internal organs nothing noteworthy was found.

As shown by Fig. 20, the rats, when fed on a diet without phenylalanine, lost  $10.0 \pm 1.7$  grams in weight during the course of 10 days. A concentration of ca. 0.4 % just sufficed for the maintenance of the original weight. The concentration of dl-phenylalanine required for maximal growth appears to lie at about 2—4 per cent. If this concentration was raised the increase in weight tended to fall off and when it rose to ca. 12 %, a complete cessation was observed (toxic effect). If the rats were fed on a diet containing 16 % dl-phenylalanine, they lost in weight to the extent of  $4.7 \pm 1.7$  grams in 10 days.

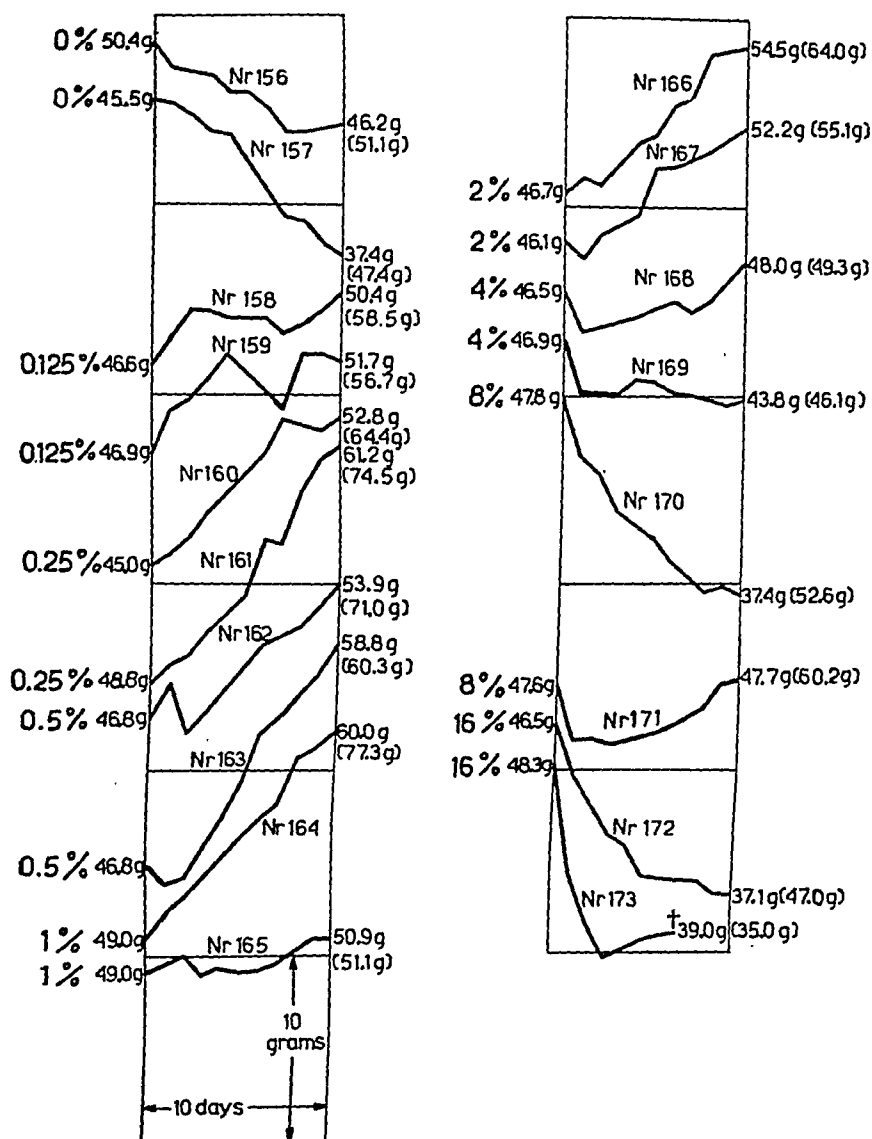


Fig. 23. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-tryptophane. — The concentrations of the dl-tryptophane are shown in front of the curves. Notation the same as in Fig. 1.

In default of dl-phenylalanine in the diet the result, as shown by Fig. 22, was a poor appetite, the consumption of food being only about  $47 \pm 5.4$  grams during the course of 10 days. On the addition of dl-phenylalanine the appetite began to improve, reaching a maximum at a concentration of 1—2%. If this concentration was raised the appetite tended to fall off, so that at

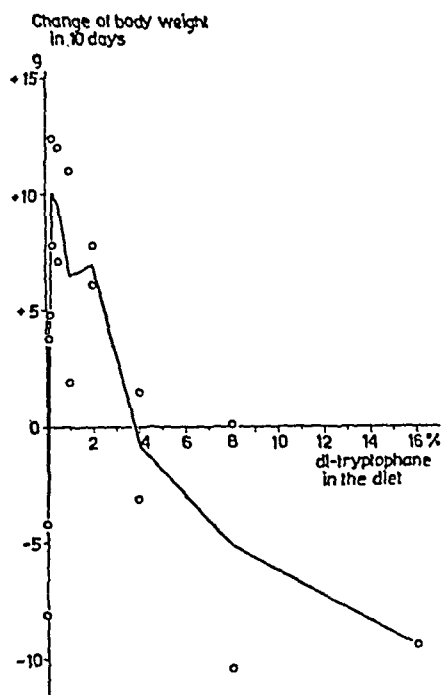


Fig. 24.

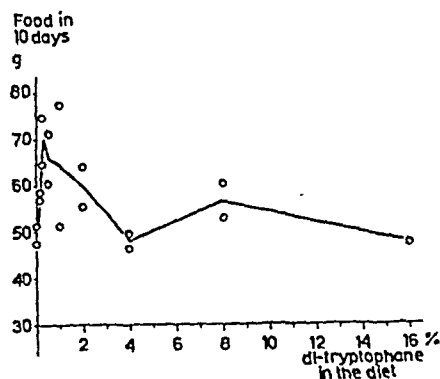


Fig. 25.

Fig. 24. Changes in weight in rats during the course of 10 days in relation to the concentration of dl-tryptophane in the diet.—Notation the same as in Fig. 3.

Fig. 25. Connection between the consumption of food and the concentration of dl-tryptophane in the diet.—Notation the same as in Fig. 4.

16 % it was somewhat less than when dl-phenylalanine was completely lacking.

### dl-Tryptophane

The concentrations of dl-tryptophane used in these tests were 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 %. The weight curves thus obtained are shown in Fig. 23. The connection between the changes in weight and the concentration of dl-tryptophane is indicated in Fig. 24. The connection between the consumption of food and the percentage of dl-tryptophane in the diet is shown in Fig. 25.

RESULT. One of the two rats fed on a diet containing 16 % dl-tryptophane died on the 8th day after a marked fall in weight (weight curve No. 173, Fig. 23). During the last few days of

the test the rat was dirty, but otherwise showed no noteworthy external symptoms. All the other rats survived and no special symptoms could be observed.

In default of dl-tryptophane, the weight of the rats fell by  $6.2 \pm 3.0$  grams in 10 days (Figs. 23 and 24). At a concentration of ca. 0.1 % the animal's original weight was maintained and at 0.125—2 % maximal growth was obtained. If the concentration was increased, the growth diminished, so that at about 4 % a cessation of growth was observed (toxic effect). At concentrations of 8 and 16 % in the diet the animals decreased in weight.

As may be inferred from Fig. 25, a poor appetite resulted from the lack of tryptophane, the consumption being merely  $49 \pm 5.8$  grams in 10 days. The appetite improved if dl-tryptophane was supplied in moderate amounts; at a concentration of 0.25—2 % the consumption of food was at its maximum. If the concentration was increased any further the appetite again tended to fall off, so that at 16 % the consumption became approximately the same as on a diet devoid of tryptophane.

### dl-Histidine

The concentrations of dl-histidine were 0, 0.25, 0.5, 1, 2, 4, 8, and 16 %.

The weight curves obtained in these tests are shown in Figs. 26 and 27. The changes in weight in relation to the concentration of dl-histidine are shown still more distinctly by Fig. 28. The variations in the appetite with different concentrations of this acid are seen from Fig. 29.

RESULT. Three rats out of four fed on a diet containing 16 % dl-histidine (weight curves 187 A, B, and C, Fig. 27) died on the 8th, 4th, and 7th day, after a moderate fall in weight. On the preceding days all were dirty, had slight diarrhea and were markedly apathetic. The other rats, apart from changes in weight, showed no noteworthy symptoms.

As may be seen from Figs. 26 and 28, on a diet devoid of histidine the rats lost  $7.2 \pm 3.1$  grams in weight during the course of 10 days. When dl-histidine was supplied the fall in weight decreased, so that at a concentration of ca. 0.2 % the

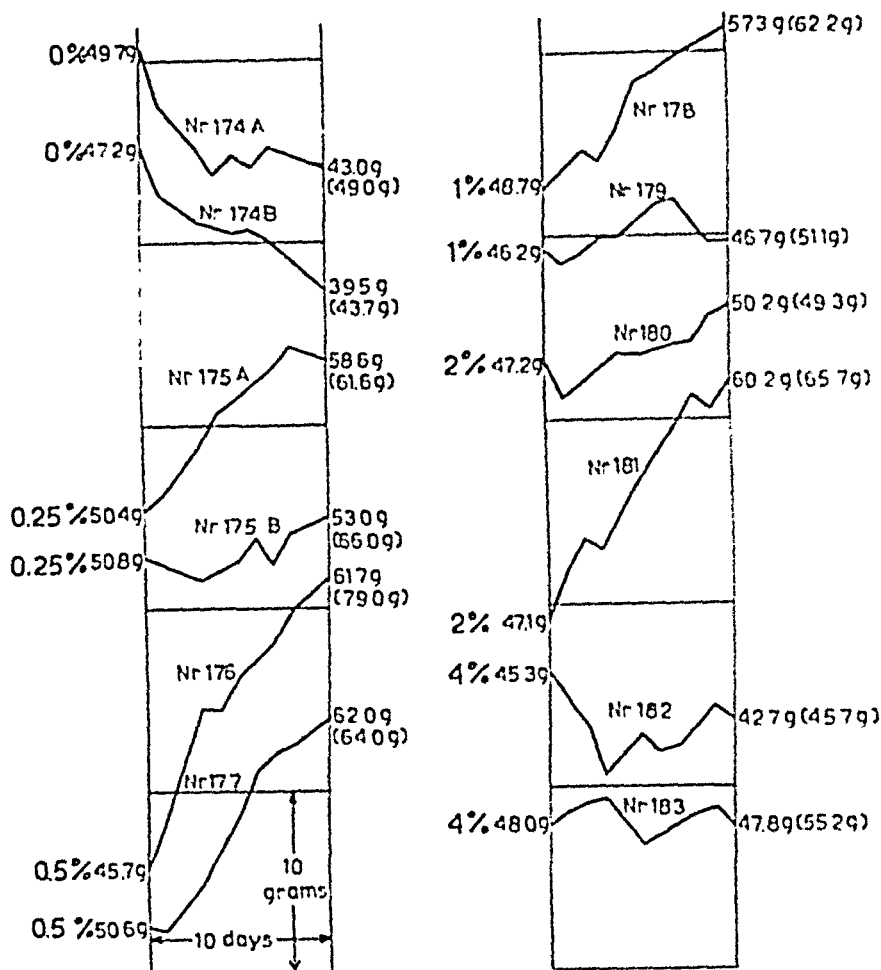


Fig. 26. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-histidine. — The concentrations of dl-histidine in the diet are shown in front of the curves. Notation the same as in Fig. 1.

weight was maintained (Fig. 28). 0.50—2 % dl-histidine was required for maximal growth. At a higher concentration the growth diminished and at concentrations of 4 and 8 % the result, broadly speaking, was a complete cessation of growth (toxic effect). A diet containing 16 % dl-histidine was fatal. As mentioned above, three of the rats died and the survivor lost 5.8 grams in weight during the 10 days of the test.

If the diet was completely devoid of histidine the appetite was bad, the consumption of food being only  $46 \pm 5.2$  grams in



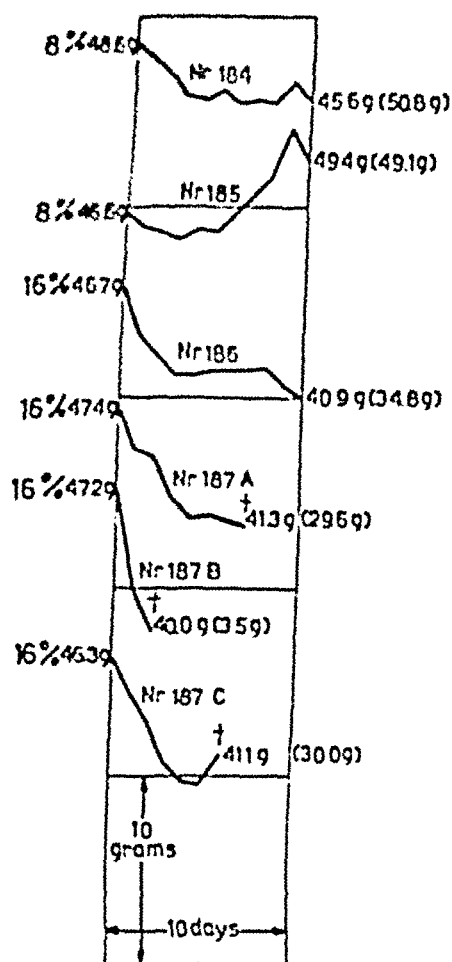


Fig. 27.

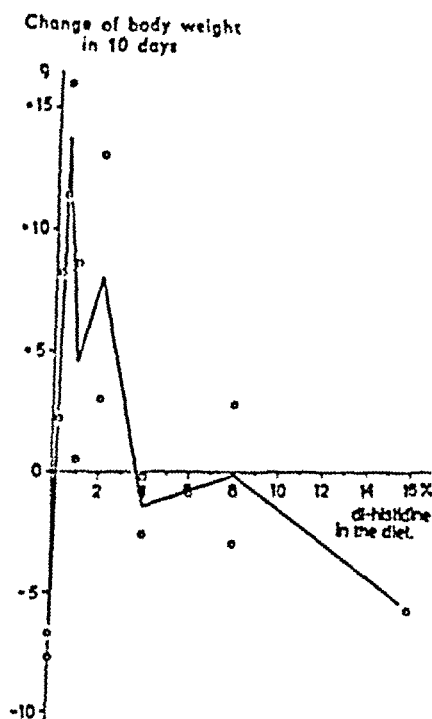


Fig. 28.

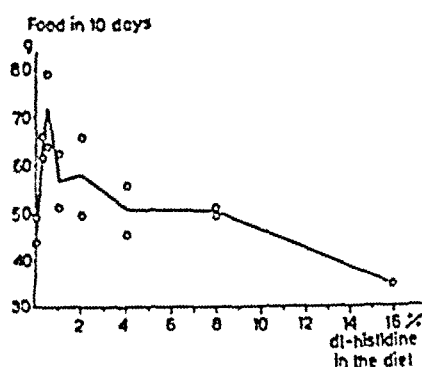


Fig. 29.

Fig. 27. Curves showing the growth of rats on a diet containing 9 synthetic amino acids and different concentrations of dl-histidine. — Notation the same as in Fig. 26.

Fig. 28. Changes in weight in rats during the course of 10 days in relation to the concentration of dl-histidine in the diet. — Notation the same as in Fig. 3.

Fig. 29. Connection between the consumption of food and the concentration of dl-histidine in the diet. — Notation the same as in Fig. 4.

10 days. As soon as dl-histidine was supplied, it began to improve, reaching a maximum at a concentration of  $0.25 \pm 0.5$  %. As the concentration was successively raised the consumption of food diminished, so that at 16 % it fell below the amount consumed on a diet devoid of histidine.

**Effect on the body-weight of protein- and amino acid-free diet, casein diet and ordinary diet.**

The effect on the body-weight of a diet containing neither protein nor amino acids was investigated. In these tests a diet (No. 198), of which the composition is shown in Table VII, was used. Moreover, growth tests were made on a diet (No. 200, Table VII) containing 15 % vitamin-free casein.

Another group of the rats tested were fed on an ordinary diet, consisting of barley grain, rye bread and milk. The conditions in these tests were the same as those previously mentioned. Before each test with ordinary diet and casein diet, the rats were kept for two days on the respective diets in order to accustom them to the change. Two rats were put on diet No. 198. The weight curves thus obtained are seen in Fig. 30 (Nos. 198 and 199). The casein diet No. 200 was given to 10 rats and the free diet to the same number. The results of these tests are found in Table VIII.

**RESULT.** It is seen from Fig. 30 that on the diet devoid of protein and amino acids the rats lost 11.8 and 13.3 grams in 10 days, and that the consumption of food was 45.7 and 43.8 grams, respectively.

As shown by Table VIII, the increase in weight during the 10 days averaged  $25.4 \pm 2.0$  grams on the casein diet and

**TABLE VII.** Composition of a diet (No. 198) devoid of protein and amino acids and of a casein diet (No. 200).

	Diet No. 198 g	Diet No. 200 g
Casein, vitamin-free		22.5
M <sub>1</sub> .....	96.5	96.5
Saccharose .....	53.5	31.0
Total	150.0	150.0

TABLE VIII. Increase in weight and consumption of food when the rats were fed on a casein diet, and increase in weight when they were allowed an ordinary diet.

Diet	Increase in weight per 10 days g	Consumption of food per 10 days g
Casein 15 % .....	21.5	78.9
	33.4	111.6
	15.2	73.7
	25.1	97.3
	18.5	79.2
	25.2	113.6
	32.5	81.7
	31.7	92.8
	21.7	75.7
	29.5	81.2
Average	25.43 $\pm$ 1.98	88.57 $\pm$ 4.62
Ordinary diet.....	14.8	
	20.0	
	25.8	
	17.5	
	20.8	
	19.2	
	15.4	
	27.3	
	27.5	
	29.5	
Average	21.78 $\pm$ 1.69	

21.8  $\pm$  1.7 grams on the ordinary diet. The consumption of food on the casein diet was 88.6  $\pm$  4.6 grams.

#### Growth with only the amino acids essential for growth

The foregoing tests indicate the most suitable concentrations of the essential amino acids for maximal growth. On the basis of these results, tests were made in order to ascertain how far the growth of rats could be maintained on an amino acid mixture containing only the nine amino acids essential for growth. The composition of the amino acid mixture used is shown in Table VI (column 11). The different amino acids were included in amounts lying within the limits of the concentrations found

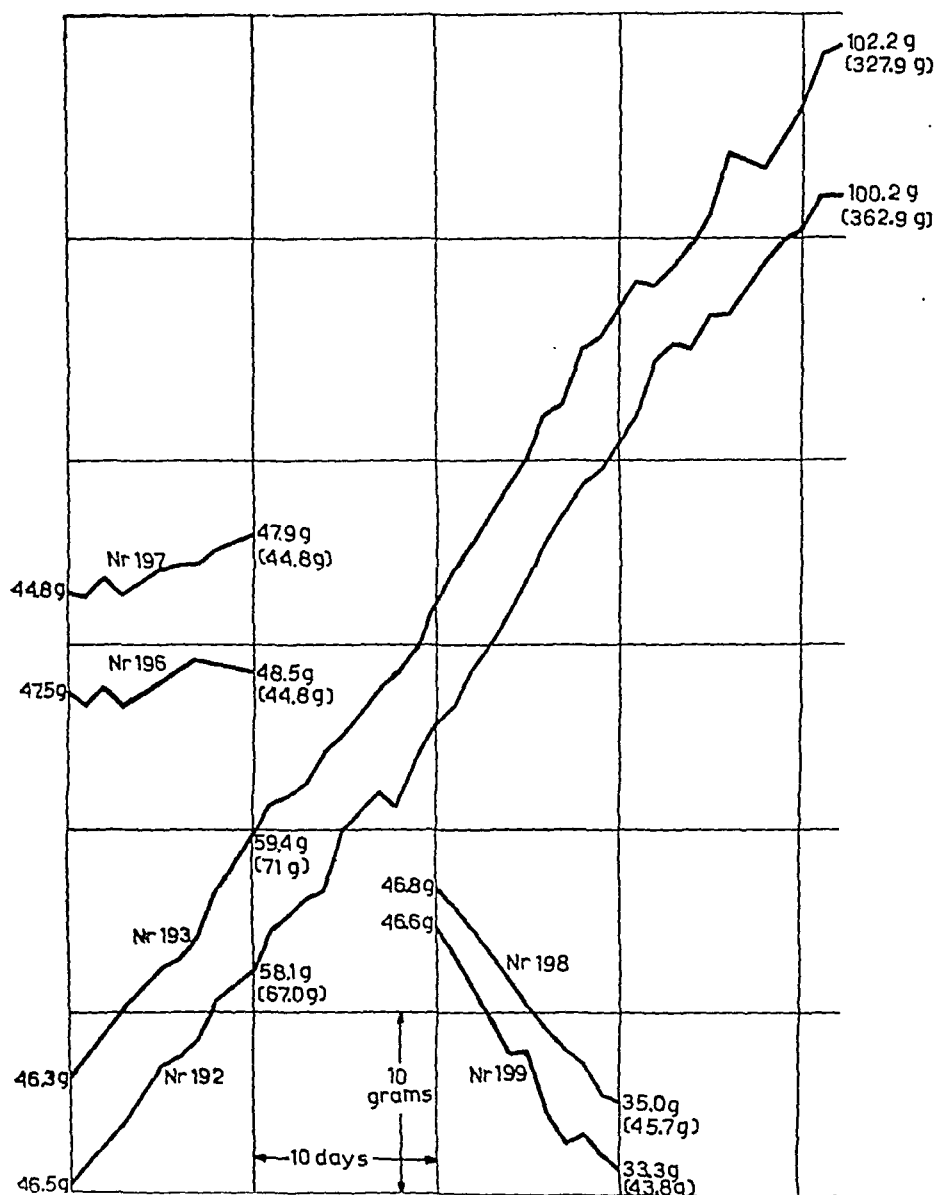


Fig. 30. Weight curves obtained in tests with diets containing 1) the nine synthetic amino acids essential for growth (Nos. 192 and 193), 2) these amino acids plus dl-arginine, the supply of food being restricted to 4.48 grams per day (Nos. 196 and 197), 3) no protein nor amino acids (Nos. 198 and 199). — Notation the same as in Fig. 1.

to be required for maximal growth. Two rats were put on this diet and the test proceeded for 42 days. The weight curves thus obtained (Nos. 192 and 193) are shown by Fig. 30.

TABLE IX. Means of changes in weight and food consumption at varying concentrations of the different amino acids.

Amino acid	Content of the amino acid in diet %	Weight change		Food consumption	
		Mean g/10 days	Standard error of mean	Mean g/10 days	Standard error of mean
dl-Histidine .....	0	- 7.20	$\pm 3.08$	46.35	$\pm 5.21$
	0.25	5.20		63.80	
	0.5	13.70		71.50	
	1	4.55		56.65	
	2	8.05		57.50	
	4	- 1.40		50.45	
	8	- 0.10		49.95	
dl-Isoleucine .....	0	-16.00	$\pm 1.04$	36.70	$\pm 3.22$
	0.5	- 2.50		55.15	
	1	7.20		74.65	
	2	8.60		67.95	
	4	9.95		66.40	
	8	11.25		62.55	
	16	3.85		49.75	
dl-Leucine .....	0	-10.60	$\pm 1.56$	42.85	$\pm 4.34$
	0.5	4.55		60.95	
	1	10.55		60.55	
	2	13.35		69.45	
	4	10.60		64.65	
	8	7.95		61.05	
	16	0.35		49.75	
dl-Lysine .....	0	- 2.95	$\pm 0.87$	45.45	$\pm 4.87$
	0.25	0.95		62.20	
	0.5	5.95		59.40	
	1	12.35		62.35	
	2	12.50		55.20	
	4	9.80		57.00	
	8	9.15		51.85	
dl-Methionine .....	0	-10.30	$\pm 1.76$	46.95	$\pm 3.99$
	0.125	- 7.10		51.45	
	0.25	10.00		67.25	
	0.5	10.95		71.65	
	1	13.55		65.85	
	2	7.10		52.15	
	4	- 4.70		36.25	
	8	- 9.55		38.40	

Amino acid	Content of the amino acid in diet %	Weight change		Food consumption	
		Mean g/10 days	Standard error of mean	Mean g/10 days	Standard error of mean
dl-Phenylalanine . . . . .	0	-10.00		47.30	
	0.25	- 4.20		50.60	
	0.5	2.90		60.20	
	1	8.25	$\pm 1.68$	69.20	$\pm 5.44$
	2	12.60		73.00	
	4	9.55		59.90	
	8	3.85		55.30	
	16	- 4.70		37.65	
dl-Threonine . . . . .	0	-10.70		45.05	
	0.25	- 7.55		42.95	
	0.5	12.00		69.95	
	1	11.20	$\pm 1.70$	72.70	$\pm 3.52$
	2	8.85		60.65	
	4	8.90		60.90	
	8	- 6.10		43.70	
	16	- 3.35		51.80	
dl-Tryptophane . . . . .	0	- 6.15		49.25	
	0.125	4.30		57.60	
	0.25	10.10		69.45	
	0.5	9.55	$\pm 2.95$	65.65	$\pm 5.78$
	1	6.45		64.20	
	2	6.95		59.55	
	4	- 0.8		47.70	
	8	- 5.15		56.40	
dl-Valine . . . . .	0	- 9.35		43.10	
	0.5	- 1.05		52.30	
	1	5.10	$\pm 1.61$	72.00	$\pm 5.81$
	2	11.20		70.65	
	4	11.25		59.55	
	8	2.30		51.95	

In each amino acid series the standard error of the mean for weight change and food consumption is given. Only the tests in which two animals had survived the experimental period are included (Calculations see page 45).

RESULT. On this diet the rats grew relatively well, increasing in weight during the first 10 days by 11.6 and 13.1 grams, respectively. The consumption of food during this time was 67.0 and 71.0 grams, respectively. It is also evident from this test that the rats can grow for at least 42 days on this diet. During

these 42 days of the test the increase in weight was 55.9 and 53.7 grams, respectively. After this 42-day period the appearance of the animals was completely normal.

### **Growth with the amino acids essential for growth and dl-arginine, supplied in limited amounts**

In the foregoing tests it was shown that, with a lack of one or other of the amino acids essential for growth, the consumption of food was poor. The daily consumption in such cases averaged 4.48 grams (estimated according to the figures in Table X, p. 80). The question thus arose how the growth would be affected if this amount of food were supplied in a diet containing the essential amino acids plus dl-arginine in optimal concentrations. Two tests of this kind were made, the animals for 10 days receiving only 4.48 grams per day of a diet containing the amino acid mixture specified in Table VI (col. 12). The weight curves thus obtained (Nos. 196 and 197) are shown in Fig. 30.

RESULT. When the animals received 4.48 grams daily of the above-mentioned diet, they showed an increase in weight of 3.1 and 1.0 grams respectively in the course of 10 days.

### **Resumé of the results**

The experiments reported in the foregoing have shown that growth in rats can be obtained on a diet in which the protein has been entirely replaced by a mixture of the synthetic amino acids essential for growth, either alone or with the addition of dl-arginine. These findings fully confirm the results of the investigations previously made by the author (WRETTLING 1948).

Varying concentrations of the amino acids essential for growth had the following effects on the body-weight:— If one of the amino acids was completely lacking, the animals lost in weight, but gained in weight when the amino acid in question was supplied. The extent of this gain in weight depended on the concentration of the amino acid. At a certain low concentration the animals remained stationary in weight (so-called maintenance concentration). An increase of the concentration up to a certain

point resulted in maximal growth. If it was raised still further, the increase in weight tended to diminish, ultimately ceasing altogether, a state termed in this paper toxic concentration. If the concentration was raised further, the result was actually a decrease in weight. Indeed, at such concentrations some of the animals died before the 10 days of the test were terminated.

The results of the tests are summarized in Table X. The decline in weight varied according to the different amino acids absent from the diet. When methionine, isoleucine or leucine were lacking some of the animals died in the course of the 10 days of the test period. As indicated by Table X, lack of lysine led to a slight fall in weight, amounting only to 3.0 grams in 10 days. This decline is considerably smaller than that caused by the absence of any other amino acid. When tryptophane or histidine were lacking, the result was a moderate decline in weight, amounting to 6.2 and 7.2 grams in 10 days respectively. The fall in weight when leucine, methionine, phenylalanine, threonine or valine were removed from the diet was approximately the same, lying between 9.4 and 10.7 grams in 10 days. The most marked reduction in weight, however (16.0 grams in 10 days), resulted when isoleucine was absent from the food. This reduction is significantly larger than for any of the other amino acids.

Table X shows that the concentrations for maintenance of equilibrium in the body weight varied between 0.1 % (dl-tryptophane) and 0.6 % (dl-isoleucine and dl-valine). The smallest concentrations required for maximal growth varied from 0.125 % (dl-tryptophane) to 4 % (dl-isoleucine). The highest concentration for maximal growth varied still more, i.e. from 1 % (dl-methionine) to 8 % (dl-isoleucine).

In order to obtain some idea of the magnitude of the maximal increase in weight with the essential synthetic amino acids plus dl-arginine as the sole source of nitrogen, the maximum mean values in the nine experimental series were determined. The figures for the corresponding single determinations are recorded in Table XI, where the average of the maximal increase in weight is shown to be 12.2 grams in 10 days.

A toxic effect resulted at a concentration of a minimum of 3 % to a maximum of 24 %. The greatest toxicity was shown by dl-



TABLE X. Summary of results of the feeding experiments on growing rats with the synthetic growth-essential amino acids in varying concentrations.

Amino acid	Loss of weight in default of stated amino acid		Concentration entailing			Consumption of food in default of stated amino acid		Concentration required for max. consumption of food %
	g/10 days	Average g/10 days	maintenance of body weight %	max. growth %	toxic effect %	g/10 days	Average g/10 days	
dl-Histidine . . . .	— 6.7	— 7.2 ± 3.1	0.2	0.5—2	4—8	49.0	46 ± 5.2	0.25—0.5
dl-Isoleucine . . . .	— 7.7	— 16.0 ± 1.0	0.6	4—8	24	43.7	37 ± 3.2	1
dl-Leucine . . . . .	— 16.4	— 10.6 ± 1.6	0.4	1—4	16	39.0	43 ± 4.3	0.5—8
dl-Lysine . . . . .	— 15.6	— 3.0 ± 0.9	0.2	1—2	16	34.4	46 ± 4.9	0.25—4
dl-Methionine . . . .	— 8.4	— 10.3 ± 1.8	0.2	0.25—1	3	38.3	47 ± 4.0	0.25—1
dl-Phenylalanine	— 12.8	— 10.0 ± 1.7	0.4	2—4	12	47.4	47 ± 5.4	1—2
dl-Threonine . . . .	— 1.8	— 10.7 ± 1.7	0.4	0.5—4	6—7	53.6	45 ± 3.5	0.5—1
dl-Tryptophane .	— 4.1	— 6.2 ± 3.0	0.1	0.125—2	4	37.3	49 ± 5.8	0.25—2
dl-Valine . . . . .	— 13.0	— 9.4 ± 1.6	0.6	2—4	16	45.9	43 ± 5.8	1—2
	— 7.6					48.0		
	— 9.9					51.2		
	— 10.1					43.4		
	— 11.2					46.4		
	— 10.2					43.7		
	— 4.2					51.1		
	— 8.1					47.4		
	— 8.3					45.7		
	— 10.4					40.5		

For calculation of the standard errors tabulated see page 45 and Table IX.

methionine; next in order came dl-tryptophane, dl-histidine, dl-threonine, dl-valine, dl-phenylalanine, dl-leucine, dl-lysine and dl-isoleucine.

As indicated by the tests with the different amino acids, the appetite of the animals varied considerably. When one of the amino acids essential for growth was lacking in the diet, the result was a poor appetite; it improved, however, when the missing amino acid was supplied, increasing to a maximum with a certain concentration of the amino acid. If the concentration was raised still further, the appetite tended to fall off and in certain cases was actually poorer than with a total absence of the amino acid from the diet.

Table X shows the consumption of food in the absence of the respective amino acid in the diet and the concentration that tended to stimulate the appetite to its maximum.

These values seem to call for the following comments. In the absence of an amino acid, with the exception of dl-isoleucine, the consumption of food was, broadly speaking, the same, varying between 43 and 49 grams. The corresponding figure in the absence of dl-isoleucine was 37 grams, and thus remarkable low. The average of all these values in default of an amino acid is  $44.8 \pm 1.2$  grams in 10 days.

The maximal consumption of food was estimated in the same way as the maximal increase in weight, the result being shown in Table XI. Thus, the maximal consumption of food was on an average  $70.8 \pm 1.5$  grams in 10 days.

The smallest concentrations required for maximal appetite varied from 0.25 % at lowest (dl-histidine, dl-lysine, dl-methionine and dl-tryptophane) to at most 1 % (dl-isoleucine, dl-phenylalanine and dl-valine). The highest concentration for maximal appetite varied between 0.5 % (dl-histidine) and 8 % (dl-leucine).

When the animals were put on a diet completely devoid both of amino acids and protein, they lost 11.8 and 13.3 grams in weight in 10 days. During this ten-day period the consumption of food was 45.7 and 43.8 grams, respectively, the average being 44.75 grams, i. e. approximately the same as the average (44.8 g) for the tests in which an amino acid was lacking.

If the rats received this amount (4.48 grams per day) of a diet containing the growth essential amino acids plus dl-arginine

TABLE XI. Max. increase in weight and consumption of food in tests with synthetic growth-essential amino acids.

	Figures for max. increase in weight g/10 days	Figures for max. con- sumption of food g/10 days
dl-Histidine .....	16.0	79.0
	11.4	64.0
dl-Isoleucine.....	11.5	70.6
	11.0	78.7
dl-Leucine.....	12.2	70.2
	14.5	68.7
dl-Lysine.....	12.3	56.8
	12.4	67.9
dl-Methionine.....	14.1	77.9
	13.0	65.4
dl-Phenylalanine ...	13.5	76.5
	11.7	69.5
dl-Threonine.....	8.1	79.2
	15.9	66.2
dl-Tryptophane ....	7.8	64.4
	12.4	74.5
dl-Valine.....	12.2	77.8
	10.3	66.2
Average	$12.24 \pm 0.52$	$70.75 \pm 1.53$

in optimal amounts, the result was a slight increase in weight, 3.1 and 1.0 grams in 10 days.

The tests also indicate that rats grew relatively well on a diet in which the protein had been completely replaced by the nine synthetic amino acids essential for growth. In the course of 42 days two rats increased in weight by 55.9 and 53.7 grams, respectively.

The increase in weight on a casein diet and ordinary diet was 25.4 and 21.8 grams respectively, in 10 days. The consumption of food on the casein diet was 88.6 grams during the same period.

## CHAPTER VI

## Discussion

ALBANESE and IRBY, in the report on their investigation (*loc. cit*), stated that in their opinion, the racemic forms were so toxic that growth could not be obtained if the essential amino acids were supplied in the dl-form. That this statement is completely misleading is shown by the experiments reported here. It can be pointed out in this connection that in the investigations made by the forementioned authors, isoleucine was supplied in the form of a "l-leucine-isoleucine mixture" and that the concentration of the isoleucine contained in it might probably be too low to produce growth; this in fact has also been pointed out by ROSE, OESTERLING, and WOMACK (1948).

The maximal increase in weight in the present experiments (diets with the amino acids essential for growth plus dl-arginine) was about  $1.2 \pm 0.05$  grams per day. If the amino acids in the diet were replaced by vitamin-free casein (15 %), a growth of  $2.5 \pm 0.2$  grams per day resulted. With animals of the same race, body weight and sex, an increase in weight of  $2.2 \pm 0.2$  grams per day was obtained if they were allowed an ordinary diet (see p. 73). There is no significant difference between these two last-mentioned figures for growth. This implies that the vitamin content in the diet given suffices at any rate for test periods of 10 days. The test diets contained no liver extract or biotin, thus indicating that for short experiments, only the vitamins used here are necessary. Thus the rate of growth on the diet containing the 10 synthetic amino acids only was about 50 per cent of the rate of growth on casein diet or ordinary diet.

One of the reasons which may contribute to less satisfactory growth than that obtained when casein is included in the diet is that the rat is unable to synthesize certain of the amino acids with sufficient rapidity. ROSE, OESTERLING, and WOMACK (1948) have shown that the growth in rats receiving only the 9 essential amino acids plus arginine is only 70—75 per cent of that obtained when 19 amino acids are supplied. This, they consider, is due to the tardy synthesis in the organism of the missing amino acids,

especially of glutamic acid. Investigations by ELVEHJELM (1948) have yielded similar results.

Another possible explanation of the poorer increase in weight is that the growth factor, streptogenin, detected by WOOLLEY (1941, 1945, 1946), is lacking in these preparations. WOMACK and ROSE (1946 a) showed that the growth with pure amino acids will be increased if part of the amino acid mixture is replaced by casein. This fact indicates the existence of some additional factor in the protein necessary for optimal growth.

A third possibility is that the optical isomer which is not utilized may have a certain toxic effect. An indication that the racemic forms have greater toxicity than the natural forms is that in experiments with intravenous injection HOWE, UNNA, RICHARDS, and SEELER (1946) have shown that mixtures containing only the natural isomers are better tolerated by dogs than the racemic forms. In their experiments it was shown that especially the dl-form of methionine was toxic for animals, whereas the l-form was well tolerated. The toxic effect of some of the other unnatural isomers must be rather slight, at any rate as regards tryptophane, histidine and arginine. BORMAN, WOOD, BLACK, ANDERSON, OESTERLING, WOMACK, and ROSE (1946) in fact show that rats (sex not mentioned) increased in weight at the rate of 1.3 grams a day on a diet including Rose's 10 essential amino acids. All of them except histidine, arginine and tryptophane were racemic forms. Since the growth was approximately the same as in the tests made by the present author, this indicates that the artificial isomers of these three amino acids are not particularly toxic. In an investigation made by ROSE, OESTERLING, and WOMACK (1948) with a mixture of ROSE's 10 essential amino acids, an increase in weight of 70—75 % of optimal increase was obtained. The amino acid mixture contained 5 natural forms. In addition to arginine, histidine and tryptophane, lysine and leucine also occurred as natural isomers, in distinction from the above-mentioned investigation by BORMAN et al. (*loc. cit.*). As ROSE and co-workers obtained a larger increase (70—75 %) in weight than that found by the present author (50 %) in his experiments, it might be supposed that this discrepancy is connected with some toxic effect of the artificial isomer of lysine or leucine, or both. It may also perhaps

be attributed to the circumstance that the major part of the saccharose in the diet had been replaced by dextrin. At any rate the toxic effect of the artificial isomer of leucine and lysine must be rather small as in the author's test it was shown that the toxicity of dl-leucine and dl-lysine is slight. The only possibility of obtaining definite information about the assumed toxicity of the unnatural isomers is to make corresponding experiments with the isomers resolved from dl-forms.

Fourthly, a possible explanation of the relatively poor growth on the diet indicated is that the consumption of food, which averages  $7.1 \pm 0.2$  grams per day, is considerably smaller than on a casein diet, when the consumption of food amounts to  $8.9 \pm 0.5$  grams per day. This may signify that certain substances required for optimal appetite are lacking in the diet, or else that the protein in itself tends to stimulate the appetite. The stimulating substances may conceivably be either glutamic acid or one of the non-essential amino acids. Presumably, WOOLLEY's streptogenin also has an appetizing effect. Further experimental investigations are necessary before a satisfactory reply can be given to this question.

\*

That the body weight of growing rats tends to be reduced on a diet from which one of the amino acids essential for growth has been removed is clearly shown in the test reported above. This tallies with investigations of earlier date. As may be seen from the weight curves, the fall in weight commences in immediate connection with the removal of one of the amino acids essential for growth. This indicates that there is no storage of the amino acids in the body. If there were such a storage, the fall in weight during the first few days of the test should be less than after the lapse of some time, when the store had been exhausted. The curves indicate exactly the reverse. The finding is borne out by the experiments of GEIGER (1947) on rats, also showing that no storage of amino acids occurs. In his experiments two different diets were prepared. The first contained all the essential amino acids but one, whilst the second contained this missing amino acid, but no others. If these diets were given alternately to rats for 12 hours each, no increase

in weight could be obtained. The amino acids tested were tryptophane, methionine and lysine. These results thus show that there is no storage of the amino acids in question nor of corresponding, but incomplete, amino acid mixtures.

Similar experiments were made by CANNON, STEFFEE, FRAZIER, ROWLEY, and STEPTO (1947). They tested two diets, one of which contained arginine, histidine, leucine, lysine and threonine, whilst the second contained the other five essential amino acids: isoleucine, methionine, phenylalanine, tryptophane and valine. If these two diets were mixed and given to protein-depleted rats, the animals ate them with avidity and increased in weight. If, on the other hand, they were given alternately for periods of different length, it was found that the appetite was poor and that the rats decreased in weight. This occurred even if the two incomplete rations were alternated at two-hour intervals (CANNON 1947). Evidently, the rat cannot store an incomplete amino acid mixture for more than two hours.

The extent of the reduction in weight in default of one of the ten amino acids essential for growth may be considered to depend on the balance between the reversible and irreversible reactions in which the amino acids take part. As reversible reactions we may consider those which involve deamination and re-synthesis by amination. Irreversible reactions are those in which the amino acids are deaminated, are consumed by combustion, enter into other metabolic processes or are excreted. Another possible explanation of the difference of the reduction in weight in default of an amino acid essential for growth is that an internal rearrangement of amino acids may take place. If, for example, a protein containing a relatively high percentage of a particular amino acid is broken down, this freed amino acid may take part in the synthesis of a protein containing the amino acid in a low concentration. The fall in weight is thus counteracted for some time.

Another matter which may contribute to the difference in reduction of weight is that certain proteins lacking one or more of the amino acids essential for growth are produced by the organism. The implication is that the body will not be deprived of this protein if one of the lacking amino acid is excluded from the diet.

The results obtained point to a considerable difference between the various amino acids as regards the effect of their absence on the weight. This effect is significantly smaller when lysine is missing in the diet. That the fall in weight will then be quite slight corresponds with the findings of several other investigators. Thus, it has been shown by BURROUGHS, BURROUGHS, and MITCHELL (1940) in experiments on white adult rats that the withdrawal of lysine or histidine from the diet will not necessarily affect the nitrogen balance — or in any case very slightly. This indicates that in full-grown rats the metabolism of lysine and histidine must be very low. NEUBERGER and WEBSTER (1945) suggest that the wear and tear quota should be applied to the different amino acids and not to protein. In such case this would mean that the wear and tear quota for lysine is particularly low. The forementioned authors show that in growing rats a diet devoid of lysine entails merely a slight decline in weight. That lysine holds a unique position among the amino acids is indicated by the studies of RATNER, RITTENBERG, and SCHOENHEIMER (1939) and of WEISSMAN and SCHOENHEIMER (1941), in which, by means of isotopes, they investigated the metabolism of the amino acids. These studies show that the amino acids constantly undergo deamination and reamination. This applies to all the amino acids with the sole exception of lysine. The metabolism of lysine differs from that of the other amino acids in that, when once deaminated, it cannot be aminated again. Apparently this deamination proceeds much more slowly in lysine than in the other amino acids.

With a lack of tryptophane or histidine in the diet, the decline in weight was somewhat more marked than if lysine was lacking. In the case of histidine, and apparently also of tryptophane, this is due to the low wear and tear quota, as indicated by the forementioned experiments of BURROUGHS, BURROUGHS, and MITCHELL (*loc. cit.*).

A third group of amino acids consists of methionine, leucine, phenylalanine, threonine and valine, where the reduction of weight in default of one of the amino acids was approximately of the same magnitude as when the animals were fed on a diet completely devoid of nitrogen. This implies that the said amino acids have about the same wear and tear quota, and that it is their



metabolic rate which determines the fall in weight when the animals are given a diet devoid of protein and amino acids.

When isoleucine was withdrawn from the diet, the fall in weight was considerably greater than when any other amino acid was missing, and even than on a nitrogen-free diet. The probable explanation is that in default of dl-isoleucine the loss of appetite was considerably greater than when any other amino acid was lacking. It may therefore be presumed that isoleucine holds a key position in the mechanism which regulates the appetite. As shown by the tests reported on p. 78, a complete diet of 44.8 grams per 10 days is required for maintenance of the weight. If dl-isoleucine was removed, the consumption of food fell to an average of 36.7 grams per 10 days, which signifies that the caloric amount supplied to the animals was 18 % less. This should suffice to explain the greater loss of body-protein in these tests.

The minimum maintenance concentration of the different amino acids is equivalent to the concentration at which no change in weight occurs when the concentration has been raised from 0 %. This maintenance concentration as well as the concentration of the amino acids required for maximal growth depends partly on the extent to which they are included in the body-proteins, partly on the above-mentioned balance between reversible and irreversible metabolic reactions. It also depends on the content of the other ingredients in the diet. Thus, the effect of methionine on the growth depends on the choline and fat content of the food. If the choline content in the diet is low and the fat concentration high, more methionine will be required. It was shown by TREADWELL (1948) that on a choline-free diet, 1—1.5 % dl-methionine is required for optimal growth and normal fat content in the liver, but that if 0.2 % choline chloride is included, 0.5 % dl-methionine is sufficient.

In the tests made by the present author, 0.2 % choline chloride was included, and optimal growth was obtained at a minimum dl-methionine concentration of 0.25 %.

A higher concentration of dl-methionine is, however, required if the concentration of nicotinamide in the diet is increased (HANDLER 1944, HANDLER and DANN 1942).

If cystine is supplied at the same time, the necessary concentration of methionine in the diet may be lower (WOMACK and ROSE

TABLE XII. Concentrations of the essential amino acids required for maximal growth of rats according to the author and ROSE (1937).

	Acc. to the author dl-form in the diet %	Acc. to ROSE (1937) l-form in the diet %
Histidine.....	0.5—2	0.4
Isoleucine .....	4—8	0.5
Leucine.....	1—4	0.9
Lysine.....	1—2	1.0
Methionine .....	0.25—1	0.6
Phenylalanine .....	2—4	0.7
Threonine .....	0.5—4	0.6
Tryptophane .....	0.125—2	0.2
Valine .....	2—4	0.7

1941). The reason is that part of the methionine will be used for the synthesis of cystine if the latter is lacking.

SINGAL, SYDENSTRICKER, and LITTLEJOHN (1947) showed that rats on a diet containing 9 % casein will increase their rate of growth if nicotinic acid or l-tryptophane are supplied. This indicates that the concentration of tryptophane required for optimal growth is dependent on the content of nicotinic acid in the food, and also that tryptophane participates in the synthesis of nicotinic acid. This implies that the optimal concentration of dl-tryptophane found in the tests made by the present author is applicable only when it stands in relation to the content of nicotinic acid.

The concentration of phenylalanine required for optimal growth may be reduced if tyrosine is supplied. It was shown by WOMACK and ROSE (1946 b) that when dl-phenylalanine was included in the diet in suboptimal amounts, a poor increase in growth was obtained, but that if l-tyrosine was supplied, optimal growth resulted. This is because, as shown by SCHOENHEIMER and MOSS (1940), tyrosine is synthesized in the organism from phenylalanine.

The concentrations of the amino acids essential for maximal growth found in the investigation reported above are compared in Table XII with the figures given by ROSE (1937). The mi-

nimum level of phenylalanine was later revised from 0.7 to 0.9 per cent by ROSE and WOMACK (1946 b).

The above figures given by ROSE hold good, however, only when non-essential amino acids are also present. When regard is paid to which isomers can be utilized by the body, it may be noted that the optimal concentrations indicated by ROSE for leucine, lysine, methionine, threonine and tryptophane lie within the range given by the author. On the other hand ROSE's optimal figures for histidine, isoleucine, phenylalanine and valine are somewhat lower than those of the author. This may partly depend on the fact that non-essential amino acids were included in ROSE's experiments.

The toxic concentration varies greatly as regards the different amino acids. The reason for their stated toxicity has not been ascertained in detail. The most plausible explanation is that there is a certain antagonism between them, as has indeed been directly shown in some cases. All the amino acids have a carboxyl and  $\alpha$ -amino group. A blocking of the protein synthesis may consequently occur when the concentration of an amino acid is abnormally high.

In several cases it has been shown that the vitamin content also has a bearing on the occurrence of toxic effects.

Among the essential amino acids dl-methionine appears to be the most toxic, as cessation of growth already begins at a concentration of 3 or 4 %. It was shown by EARLE, SMULL and VICTOR (1942) that diets containing 6.4, 10 and 12.4 % dl-methionine brought about a marked fall in weight in rats and that most of them died within a week. The chief morphological change was extreme atrophy of the liver and the liver cells.

The above-mentioned experiments of HOWE, UNNA, RICHARDS and SEELER (*loc. cit.*) indicate that the toxic effect of methionine is particularly noticeable in its dl-form. That the toxicity of dl-methionine is partly due to the content of vitamin B<sub>6</sub> in the diet is shown by the experiments of SARMA, SNELL and ELVEHJEM (1947) on the growth of rats on a diet deficient in this vitamin. If 1 % dl-methionine was added, the growth was only 6 grams per week as against 18 grams without such addition. But if the diet was supplemented with 250  $\gamma$  pyridoxine HCl per 100 grams, no such reduction in weight resulted. This

corresponds with the results obtained by MILLER and BAUMANN (1945), showing that on a diet containing 60 % casein, mice require three times the amount of vitamin B<sub>6</sub> as on a 20 % diet. In the tests made by the author, the rats were fed on a diet containing 500  $\gamma$  pyridoxine HCl per 100 grams. Had this amount been increased, the concentration for the toxicity of dl-methionine would presumably have been increased. This also applies to dl-tryptophane (SARMA, SNELL, and ELVEHJEM *loc. cit.*).

MARTIN (1946) has shown that dl-tryptophane is more toxic if given to rats in excess (20 % in the diet) when the supply of riboflavin is insufficient. That tryptophane in higher concentrations (20 %) is toxic, tends to reduce the weight and leads to parenchymatous degeneration of the renal tubules had been previously shown by SULLIVAN, HESS and SEBRELL (1932).

That there is a certain antagonism between tryptophane and other amino acids in regard to growth is indicated by the experiments of SINGAL, SYDENSTRICKER and LITTLEJOHN (1947) referred to above. In these tests it was shown that, if a mixture of histidine, valine, threonine and lysine, or a similar mixture without lysine, were added to the 9 % casein diet, the growth would be smaller. On the addition of l-tryptophane normal growth was obtained. There must thus be some antagonism between tryptophane and one or more of the amino acids histidine, valine and threonine.

As regards dl-phenylalanine, it should be noted that, as shown by NIVEN, WASHBURN and SPERLING (1946), its toxicity is also dependent on the concentration of tryptophane and nicotinic acid. The investigators made their tests on rats fed on a 10 % casein diet. They found that if 2 % phenylalanine were added, the increase in weight completely ceased. This effect, however, could to some extent be obviated by supplying nicotinic acid or tryptophane.

In respect of the toxicity of lysine, it was indicated by the above-reported tests that a distinct toxicity appeared at a concentration of 16 %. This is in good agreement with the above-mentioned investigations made by SULLIVAN, HESS and SEBRELL (*loc. cit.*) as well as those of LILLIE (1932), indicating that rats on a diet containing 20 % lysine are liable to show morbid changes (reduction in weight, parenchymatous and fatty degenera-

tion of the renal tubules as well as diffuse fatty degeneration of the liver).

The evidence reported shows that the toxic effect of an individual amino acid is not due solely to its concentration. Other important factors are the vitamin concentrations and the content of the other amino acids in the diet. In the light of this statement, it may be seen that the toxic concentrations referred to above hold good only when they stand in relation to the content of the other amino acids and of the different vitamins.

It was found in all the tests that the appetite depended on the concentration of the individual amino acids. That the appetite falls off on the removal of an essential amino acid has been shown, for example by FRAZIER, WISSLER, STEFFEE, WOOLRIDGE and CANNON (1947) in experiments on rats. They fed rats (weighing 220—244 grams) on a diet with 16 amino acids. If one of the essential amino acids was removed, the rats lost their appetite, with a resulting decrease in weight. It was found that if l(+)-lysine was administered subcutaneously twice a day, a good appetite and a normal increase in weight were maintained. That rats lose their appetite when one or other essential amino acid is lacking in the diet has also been pointed out by ROSE (1931, 1938).

As shown in the publications of ROSE and associates (ROSE, HAINES and JOHNSON 1942, ROSE, HAINES, JOHNSON and WARNER 1943, and ROSE, 1947), the conditions are the same in man. If one of the essential amino acids is withdrawn, a negative nitrogen balance will result. The experimental subject will then feel indisposed and lose his appetite. When the missing amino acid is again supplied, his appetite will be restored.

The loss of appetite on the withdrawal of an amino acid does not, however, suffice to explain the fall in weight. The forementioned tests in fact indicate that the consumption of food fell on an average from 7.07 grams to 4.48 grams per day if one of the amino acids essential for growth was withdrawn. This corresponds, for a rat weighing 35—50 grams, to a supply of about 17 Cal., which, according to CAMPBELL and KOSTERLITZ (1948) should suffice for the maintenance of body weight. The present author has also shown by experiment that rats increase somewhat in weight when only 4.48 grams daily of a diet con-

taining all the amino acids essential for growth, plus arginine was supplied. The consumption of food was thus reduced to about the caloric minimum. This experiment indicates that the fall in weight is not due to loss of appetite but to deficient protein synthesis in the body itself owing to lack of one or other of the amino acids which the body cannot synthesize in sufficient amounts.

This loss of appetite in default of one of the amino acids may conceivably, as is sometimes supposed, be connected with gustatory sensations. In that case, as is pointed out by FRAZIER, WISSLER, STEFFEE, WOOLRIDGE and CANNON (1947), we should be forced to the conclusion that if one of the nine amino acids supposed to be indispensable for the sensation of taste should be missing, the taste would not be whetted by the others. This, they remark, conflicts with the fact that if lysine is administered parenterally (thus outside of the mouth), a normal appetite will result even if lysine should be lacking in the diet. A more probable explanation, they think, is that the loss of appetite is due to a general disturbance of the metabolism, which has such a weakening effect on the animal and human organism as temporarily to abolish the craving for food.

The concentrations requisite for maximal growth and maximal stimulation of appetite are the same in the tests with dl-methionine; dl-isoleucine stimulates the appetite at a distinctly lower concentration than that entailing maximal increase in weight. In tests with dl-histidine, dl-phenylalanine and dl-valine the appetite is stimulated at a somewhat lower concentration than that required for maximal increase in weight.

The concentrations requisite for maximal stimulation of appetite lie within the range of those required for maximal growth in the tests with dl-threonine and dl-tryptophane. In the tests with dl-leucine and dl-lysine on the other hand, it was found that the concentrations for maximal growth lie within the range of those for maximal appetite.

The fact that the maximal increase in weight and the maximal consumption of food in some tests do not quite coincide indicates that the change in weight is due partly to the appetite and partly to the concentration of the amino acid in question.

## CHAPTER VII

## Summary

The author suggests a new division of the amino acids into three groups termed essential for growth, essential for optimal growth and non-essential. The amino acids essential for growth mean those absolutely necessary for growth, whilst amino acids essential for optimal growth signify those necessary only for optimal growth. The non-essential acids have no effect at all on the growth.

For the rat the amino acids essential for growth are: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane and valine.

The amino acids essential for optimal growth are arginine and glutamic acid. The other amino acids appear to be non-essential for the rat.

The investigation reported was intended to show the possibility of obtaining growth in rats on a diet where the protein had been replaced by a mixture consisting only of the said nine essential amino acids, *synthetically* produced. In addition, the different essential amino acids were studied, in varying concentrations, with respect to their effect on the body weight and consumption of food.

The tests were made on growing white female rats (weighing 43—51 grams). The amino acid-free part of the diet was composed of saccharose, cellulose, salt, cod liver oil, wheat-germ oil as well as the crystalline vitamins: aneurin, riboflavin, nicotinamide, pyridoxine, Ca-d-pantothenate, inositol, choline chloride, p-aminobenzoic acid, 2-methyl-1,4-naphthoquinone and folic acid. The amino acid- and vitamin-free part of the diet contained only 0.012—0.019 % nitrogen.

The experimental periods as a rule had a duration of 10 days.

The results of this investigation were:—

1. Growth in rats could be obtained if the protein in the diet was completely replaced by a mixture of the synthetically produced amino acids dl-arginine, dl-histidine, dl-isoleucine, dl-leucine, dl-lysine, dl-methionine, dl-phenylalanine, dl-threonine, dl-

tryptophane, and dl-valine. A maximum increase in weight averaging 1.2 grams per day was the result in this case.

2. Even if dl-arginine was excluded, an increase in weight of 1.2 grams per day was obtained. On such a diet, thus containing only the nine synthetic amino acids essential for growth, two rats increased in weight in the course of 42 days by 55.9 and 53.7 grams, respectively.

3. If any of the amino acids essential for growth was removed, a marked fall in weight resulted. In default of lysine, the decline in weight was much smaller (3.0 grams in 10 days) than when any of the other amino acids was withdrawn. As regards the latter, the decline in weight in 10 days (reckoned in the order of magnitude) due to the absence of the several amino acids was approximately as follows:— tryptophane 6.2 grams, histidine 7.2 grams, valine 9.4 grams, phenylalanine 10.0 grams, methionine 10.3 grams, leucine 10.6 grams, threonine 10.7 grams, and isoleucine 16.0 grams.

Some of the animals fed on a diet devoid of methionine, isoleucine or leucine, died within 10 days with symptoms of cachexia.

The decline in weight on a diet completely devoid of protein and amino acids amounted to about 12.5 grams in 10 days.

4. The changes in weight depended on the concentration in the diet of the individual amino acids essential for growth. As the concentration rose from 0 %, the weight ceased to fall and then gradually increased, reaching its maximum within certain definite concentrations, varying for the different amino acids. If the concentration was raised beyond the indicated limits, the result was a diminution in the growth or a fall in weight. The concentrations required for maintenance and maximal increase of the weight as well as those having a toxic effect were determined in regard to the different synthetic essential amino acids.

It was found that the so-called maintenance concentration was lowest for dl-tryptophane (ca. 0.1 %). Next in order came dl-histidine, dl-lysine, and dl-methionine at a concentration of about 0.2 %. For dl-leucine, dl-phenylalanine and dl-threonine a concentration of ca. 0.4 % was required, for dl-isoleucine and dl-valine 0.6 %.

The concentrations requisite for maximal increase in weight



were: dl-histidine 0.50—2 %, dl-isoleucine 4—8 %, dl-leucine 1—4 %, dl-lysine 1—2 %, dl-methionine 0.25—1 %, dl-phenylalanine 2—4 %, dl-threonine 0.5—4 %, dl-tryptophane 0.125—2 %, and for dl-valine 2—4 %.

A toxic effect on the growth resulted at the following concentrations: dl-histidine 4—8 %, dl-isoleucine 24 %, dl-leucine 16 %, dl-lysine 16 %, dl-methionine 3 %, dl-phenylalanine 12 %, dl-threonine 6—7 %, dl-tryptophane 4 %, and dl-valine 16 %.

5. The consumption of food depended on the concentration of the different amino acids in the diet. In default of one of the amino acids essential for growth the appetite was poor. When the missing amino acid was supplied, the appetite improved and at certain definite concentrations reached its maximum. If however the concentration was raised any further, the appetite deteriorated.

The consumption of food per 10 days in default of one of the amino acids, reckoned in the order of magnitude, was the following:— Isoleucine 37 grams, leucine 43 grams, valine 43 grams, threonine 45 grams, lysine 46 grams, histidine 46 grams, methionine 47 grams, phenylalanine 47 grams, tryptophane 49 grams. On an average, the consumption of food was 44.8 grams per 10 days in lack of any of the amino acids.

The concentrations required for maximal consumption of food were:— dl-histidine 0.25—0.5 %, dl-isoleucine 1 %, dl-leucine 0.5—8 %, dl-lysine 0.25—4 %, dl-methionine 0.25—1 %, dl-phenylalanine 1—2 %, dl-threonine 0.5—1 %, dl-tryptophane 0.25—2 % and dl-valine 1—2 %. The maximal consumption of food in these tests averaged 70.8 grams in 10 days.

6. The reason why only 50 per cent of the optimal growth on a casein or ordinary diet was obtained in these feeding tests is discussed. It appears to be mainly due to the lack of certain of the non-essential amino acids, especially glutamic acid, the absence of streptogenin, possibly to a toxic effect of the artificially produced isomers and to the relatively poor appetite.

7. The remarkably small decrease in weight when lysine was missing in the diet is discussed. It appears to be mainly due to the slower combustion of lysine in the body than that of the other amino acids.

8. Some aspects of the relations of the amino acids to each other and to the vitamins are discussed.

\*

The methods adopted for the syntheses of the amino acids essential for growth and of dl-arginine are described.

The following new method and procedures were elaborated:—

dl-Arginine HCl was produced by treatment with aniline in an alcoholic solution containing an excess of hydrochloric acid, in which process dl-arginine HCl, which is insoluble in alcohol, was precipitated.

In connection with PYMAN's method for the synthesis of dl-histidine, a simpler procedure for the preparation of 4-hydroxymethylglyoxaline from diaminoacetone was elaborated. By treatment of the diaminoacetone with potassium thiocyanate, 2-thiol-4-aminomethylglyoxaline was obtained. The latter product was oxidized, without isolation, into 4-hydroxymethylglyoxaline with nitric acid. Via the pierate, the hydrochloride of 4-hydroxymethylglyoxaline was obtained.

SNYDER and SMITH's method for the production of ethyl acetaminomalonate was modified by carrying out the acetylation in an ether solution, a procedure which facilitates its crystallization.

The free base of dl-histidine was produced by a new method, based on the removal of the hydrochloride in dl-histidine diHCl by treatment with aniline in an alcoholic solution where the dl-histidine is sparingly soluble and the aniline chloride readily soluble.

For dl-leucine a new technique (indicated in principle by FISCHER and SCHMITZ) was elaborated. In accordance with this technique, dl-leucine was produced from isobutyl alcohol via isobutyl bromide, ethyl isobutylmalonate, isobutylmalonic acid,  $\alpha$ -bromo- $\alpha$ -carboxyisocaproic acid and  $\alpha$ -bromoisocaproic acid, which was converted by amination to dl-leucine.

dl-Lysine, which has not previously been described in a free form, was obtained from dl-lysine HCl by removal of the hydrochloride with silver carbonate. The free base was precipitated from an alcoholic solution with ether.

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THE CYTOCHEMICAL CORRELATE  
OF MOTOR NERVE CELLS IN  
SPASTIC PARALYSIS

BY

INGRID HOCHBERG AND HOLGER HYDÉN

KAROLINSKA INSTITUTET  
STOCKHOLM





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From the Medical Nobel Institute, Department of Cell Research,  
Karolinska Institutet, Stockholm.

# **The Cytochemical Correlate of Motor Nerve Cells in Spastic Paralysis**

by

*Ingrid Hochberg and Holger Hydén*

## **The Problem**

The aim of these experiments was to induce distinct, reversible or irreversible decreases in neuronal function and to study the chemical correlate in the nerve cells affected by means of microspectrography in the ultraviolet and other cytochemical methods. Functional disturbances in the form of spastic paralysis and changes in the cells were brought about through experimentally induced ischaemia in the lumbar part of the spinal cord by means of bloodless occlusion of the abdominal aorta.

## **Scope of the Investigation**

The large motor anterior horn cells in the spinal cord were chosen as experimental material. Their size, general organization and well known cytology and chemical composition make them eminently suitable for investigations using cytochemical methods. Their cytoplasm contains large concentrations of ribose nucleic acids and proteins, and the nuclear system which directs the production of these substances is well developed. A considerable consumption of nucleoproteins takes place in connexion with motor function. On intense motor stimulation, the system for the production of nucleoproteins in the cytoplasm does not appear able to compensate the

substantial consumption and a considerable decrease of these substances in the cytoplasm results. Regeneration nevertheless starts immediately after the cessation of the intense motor stimulation and the original content and distribution of nucleoproteins in the cytoplasm is restored 48 hours after the end of the experiment (HYDÉN 1947).

With a total inhibition of function such as takes place in infection of the motor nerve cells by neurotropic viruses, the nucleoprotein production in the cytoplasm ceases (CASPERSSON and HYDÉN 1945, HYDÉN 1947).

The data concerning the motor ganglion cells given in the foregoing form the immediate background to the experiments reported in the present paper. In the experiments with ischaemia of the spinal cord during 15—25 minutes, it was possible to obtain a series of extremely distinct changes in neuronal function. It appears from a large number of earlier reports on the effects of temporary ischaemia of the spinal cord (v. the following: Historical) that this can be accompanied by a paralysis of both the spastic and the flaccid type. HÄGGQUIST (1937, 1938) found that ischaemia during 15 minutes could result in residual spastic paralysis. The large root cells and their coarse neurites are destroyed in connexion with such experiments, whereas the small intermediate cells and the fine-calibered fibres in the anterior roots are unaffected. On the basis of his findings of nerve endings "en grappe" and "en plaque" in various types of musculature, HÄGGQUIST concludes that the small intermediate cells and their fine-calibered neurites convey the impulses maintaining the tonus of the muscles. After more lengthy ischaemia, the latter cells and the fine-calibered fibres are also destroyed and flaccid paralysis develops. The changes in muscular tonus in such experiments run a course from normal, *via* spasticity and hypotonia to atonia in flaccid paralysis (REXED 1940).

*Experimentally induced ischaemia of the spinal cord thus affords a possibility of studying the chemical correlate of a series of reversible and irreversible functional stages in the motor neurons.*

## Historical

The effect of ischaemia on the central nervous system has been studied by many workers, the first experiments being carried out by NIELS STEENSEN in 1667. Studies of the effect of ischaemia on the cytological

picture in the motor nerve cells of the spinal cord are of main interest in the present investigation. For an extensive historical review of the cytological and physiological changes caused in STEENSEN's experiments, see TUREEN (1936), HÄGGQVIST (1937, 1938), and REXED (1940).

In the majority of cases experiments were carried out by means of occlusion of the abdominal aorta, either through surgical intervention or by compression against the spinal column. Duration of the occlusion has nevertheless proved to be a very uncertain factor. Thus, ROTHMAN (1899) demonstrated that ligation of the superior mesenteric artery did not altogether cut off the circulation, which was maintained through the intercostal arteries by means of the anterior spinal arteries.

HÄGGQVIST (1937, 1938) demonstrated that the large motor root cells and the coarse nerve fibres emanating from them are those types of cells that are most rapidly destroyed after ischaemia for 15 minutes. Spastic paralysis in the hind legs of the animal occurred in connexion with the experiment. In addition, KROGH (1944, 1945) was able to show that the motor nerve cells in various sections of the anterior horn vary in their sensitivity to ischaemia. The cells in the periphery of the horn are more sensitive than those nearer to the central canal.

Amongst earlier writers who have described cytological changes after ischaemia of the spinal cord, we can mention HALLER (1762), STANNUS (1852), EHRLICH-BRIEGER (1884), SINGER (1887), HERTER (1889), WIENER and MÜNZER (1895), SARBO (1895), MARINESCO (1896), JULIUS-BERGER (1896), DE BUCK and DE MOOR (1901), BÄLLET and DUTILLE (1901), GOMEZ and PIKE (1909). Amongst more recent works we have those of GILDEA and COBB (1930), TUREEN (1936, 1938), and MORRISON (1946).

The changes described affect the cytoplasm (GOMEZ and PIKE, MORRISON, MÜNZER-WIENER, TUREEN, and others) or the nucleus and the nucleolus (GOMEZ and PIKE, MORRISON, TUREEN, and others).

The first cytological signs of cell changes were observed by the different writers at very varying times after the occlusion. Thus TUREEN described cells with strongly stainable cytoplasm after 15 minutes and distinctly stainable dendrites seven hours after the experiment. MORRISON (1946) found pale cells with a disappearance of the Nissl substance, oedema and vacuolization of the cytoplasm after repeated exposure to anoxia.

The nucleolar changes described by TUREEN, MÜNZER-WIENER, and others are of particular interest for the present investigation. The fore-mentioned writers reported an enlargement of the nucleolus in a large number of cells.

GOMEZ and PIKE nevertheless state that they found no changes in the size or the stainability of the nucleolus following ischaemia of the spinal cord.

Changes in the nucleus have mainly been described as a displacement towards the periphery and an increased stainability (MORRISON, TUREEN).

A description is given in the following of TUREEN's experiment with occlusion of the abdominal aorta in the cat for 15 minutes, since his

experiment is very similar to part of our own. Flaccid paralysis started during the period of post-operative stupor and one hour afterwards spasticity set in with clonic spasms in the extremities and an accentuation of the tendon reflexes. Paralysis lasted only 5—24 hours after the experiment but the muscular spasticity remained for many days.

At the end of the ischaemic period he found no changes. After 7 and 28 hours the nerve cells stained deeply and were chromatolyzed. After the latter period the nucleoli appeared enlarged. After 36 and 48 hours, the stainability of the cells was still strong both in the nucleus and in the cytoplasm. The nucleoli also stained intensely and were often excentrically placed. TUREEN also described glia changes and tissue reactions, most pronounced 48 hours after ischaemia. After 72 hours the nucleus stained intensely, whereas the stainability of the nucleolus was normal and that of the cytoplasm stronger than normal. Five days after the experiment the nucleolus was stated to be enlarged and the borderlines of the nuclear membrane indistinct. Strongly stainable restored Nissl granules could be observed in the cytoplasm.

During the 6th and 7th days the majority of the cells had a normal appearance but seemed to stain more intensely. The nerve cells examined 14, 16, and 21 days after the experiment were normal in appearance.

It is evident from the results that paralysis occurred before the changes in the nerve cells were visible and that the severe cytological lesions were most pronounced when it had regressed (7—72 hours after the end of the experiment). This incongruity between the changes in the cells and the functional disturbances was described earlier by BALLET and DUTILLE (1897) and by ROTHMAN (1899).

TUREEN (1938) and KUCZYNSKI (1938) studied the ash content of nerve cells after occlusion. The distribution of ash in normal cells appeared to be in good conformity with the distribution of the Nissl substance. A considerable quantity of ash was obtained from the nucleolus. TUREEN describes a striking difference in the ash content after occlusion for 15 minutes and after more than 20 minutes. In the latter case the lesions were irreversible. During earlier stages of the experiment, the ash was a brownish colour, but it assumed a bluish tone in the restored cells during the 6th and 7th days. Normal ash conditions were restored after the 9th day.

In animals in which occlusion of the abdominal aorta had lasted for more than 15 minutes, the cells showed a progressive loss of ash after an initial stage with a very high content. In animals with ischaemia during 15 minutes' occlusion the opposite occurred, i. e. an initial stage of decrease was followed by a stage with an increase in the ash content.

EINARSSON, in a series of investigations, studied the nerve cells and their stainability under different experimental conditions with the use of his own staining method with galloxyanine-potassium chromic sulphate (EINARSSON 1932, 1933, EINARSSON 1946, EINARSSON and BENTSEN 1939, EINARSSON 1945). He then drew conclusions concerning the functional state from the varying stainability of the cytoplasm, since he

considered the Nissl picture — as it appears with his method — to be a coagulation model which reflects the conditions *in vivo*. Mention will only be made here of his investigation on the effect of vitamin E deficiency on the nerve cells and the changes in the nerve cells in the spinal cord with contracted or relaxed extensor muscles. On the basis of these experiments (EINARSSON and RINGSTEDT 1938), EINARSSON (cf. further 1945) correlated slight chromophilia with commencing activity, chromophobia with activity during a longer period and extreme chromophilia with inhibited functional activity. He considered extreme chromophobia to be characteristic of "central depression through fatigue".

KABAT and KNAPP 1944 experimentally produced muscle spasms in dogs by arresting the spinal cord circulation for 45 minutes and studied the pathological changes in the spinal cord. They also studied muscle spasms of the same type in patients suffering from poliomyelitis. Many muscles in these cases had a normal chronaxia, others a greatly increased chronaxia, suggesting anterior horn cell destruction. They therefore concluded that there was no correlation between anterior horn cell destruction and the muscle spasms.

In the dogs after arrest of the spinal cord circulation they found changes in the small internuncial cells but no changes in the anterior horn cells.

They therefore suggested that a lesion of internuncial neurons produces a localized muscle spasm on the basis of the release of anterior horn cells from inhibition.

## Material and Procedure

In order to avoid excessive length in the present report, we have chosen to describe our material and results in four typical groups, assembled in the following tables.

Altogether 28 rabbits — of which seven were used as controls — each weighing 2—3 kg were used. The experimental animals were divided into four groups according to the period of the experimental ischaemia and also with regard to the resulting symptoms. Slight spastic paralysis was obtained in 3 rabbits after ischaemia of the spinal cord for 15 minutes (v. table I). Pronounced spastic paralysis occurred in 11 rabbits after the same duration of ischaemia (table II) and strongly developed spastic paralysis in 4 rabbits after ischaemia for 25 minutes (table III).

The animals were killed at varying times after the end of the experiments, either immediately or up to 30 days after the occlusion.

In order to induce ischaemia of the spinal cord in the rabbits, the technique for bloodless occlusion of the abdominal aorta elaborated



Table I. *Slight Spastic Paralysis After Short Occlusion*

Number of animals	Occlusion (minutes)	Survival period	Time until development	Comments	Symptoms at death
1	15	Killed immediately after occlusion	—	—	—
1	15	1 hr.	—	The animal anaesthetized during survival period	No symptoms
1	15	2 hrs.	—	— » —	No symptoms
1	15	3 hrs.	2 hrs. 45 mins.	Slight spastic paralysis in the hind legs	Remaining spastic paralysis in the hind legs
1	15	5 hrs.	3 hrs.	Slight spastic paralysis in the hind legs	Remaining spastic paralysis in the hind legs
1	15	12 hrs.	3 hrs. 30 mins.	Slight spastic paralysis in the hind legs	Remaining spastic paralysis in the hind legs

Table II. *Pronounced Spastic Paralysis After Short Occlusion*

Number of animals	Occlusion (minutes)	Survival period	Time until development of paralysis	Comments	Symptoms at death
1	15	4 hrs.	2 hrs.	Fairly strong spastic paralysis	Remaining spastic paralysis, fairly pronounced
2	15	7 hrs.	6 hrs. 30 mins.	Spastic paralysis in the hind legs	Remaining spastic paralysis, fairly pronounced
2	15	24 hrs.	12 hrs.	Spastic paralysis in the hind legs	Remaining spastic paralysis
	15	48 hrs.	—	Spastic paralysis in the hind legs for some hours	Normal motility and tonus in the hind legs
3	15	72 hrs.	—	Spastic paralysis for 24 hrs.	Remaining muscular hypertonus in the hind legs
1	15	96 hrs.	5 hrs.	Strong spastic paralysis for 42 hrs. Ceased for 24 hrs. and reappeared for 8 hrs.	Remaining spastic paralysis
1	15	6 days	6 hrs.	Strong spastic paralysis after 48 hrs.	Remaining spasticity in the hind legs

Table III. Pronounced Spastic Paralysis After Long Occlusion

Animals	Occlusion (minutes)	Survival period	Comments	Symptoms at death
I	25	8 hrs	Spastic paralysis in the hind legs. Obvious paraesthesiae in the fore legs	Spastic paralysis in the hind legs
I	25	2 days	Spastic paralysis in the hind legs. In the fore legs paralysis in extension. Incontinentia vesicae et alvi	Spastic paralysis in the fore and hind legs
I	25	6 days	Spastic paralysis in the hind legs. Incontinentia vesicae et alvi developed immediately	Spastic paralysis in the hind legs. Incontinentia vesicae et alvi
I	25	1 month	Spastic paralysis in the hind legs	The right hind legs in extension with atrophied muscles. The left leg with spastic paralysis

by HÄGGQVIST (1937, 1938) was used. Pernoxon or nembutal anaesthesia was employed. The abdominal aorta was occluded with a metal clamp under the last rib and above the origin of the renal arteries until the pulse in the arteries of the extremities had stopped. This was controlled continuously. The total absence in the rabbit in the majority of cases of collateral connexions explains the complete ischaemia after a short time. There is, nevertheless, a certain physiological variation with the possibility of collateral connexions through the anterior spinal and posterior spinal arteries. This explains the varying results. In order to assure ischaemia and a control of the degree of circulation; TUREEN (1936) worked out a method with the injection of dye and subsequent histological control of its distribution in the spinal cord. Light has been thrown on the problem of the sensitivity of the various cell groups in the anterior horn to ischaemia by the investigations of KROGH (1944, 1945). KROGH found that the motor nerve cells in the periphery of the anterior horn, situated at the arterial endings of the capillaries, were the most resistant. The central groups of cells, which are nearer to the venous end of the capillaries, were far more sensitive. In our experiments we have studied the large nerve cells in the antero-lateral nucleus of the anterior horn, which thus correspond to the peripheral cells described

by KROGH. Our reason was that this group of cells has been studied earlier and the physiological variations in their chemical composition determined with cytochemical methods (HYDÉN 1943 b, HYDÉN and HARTFLIUS 1948).

The animals were killed either by severing the carotid arteries or by the creation of an air embolus. Small pieces of the spinal cord were excised and fixed according to the ALTMANN-GERSH freezing-drying method, in Carnoy's solution and in Stieve's mercuric chloride-formaldehyde-acetic acid solution. They were embedded in paraffin and sections cut of 5—10  $\mu$  in thickness.

The thickness of the sections was calculated in two ways. According to the first method (DORN 1935) the distance between the cover-glass and the slide is calculated by means of the interference determination. According to the other (elaborated by CASPERSSON) the actual distance between small cell structures on the upper and lower surfaces of the section is measured by a system of panchratic lenses inserted in a phase-microscope.

For photography in the ultraviolet range and for microspectrographic measurements, the sections were mounted on quartz slides in glycerol (spec. density 1.22). They were previously passed through chloroform for 5 minutes and absolute alcohol for 5 minutes.

Suitable cells were selected in the Köhler ultraviolet microscope and photographed at 2,570 Å. The cells taken for absorption measurements were freed from tissue on one side in order to obtain free space in the vicinity of the cell. Dissection was performed with small glass needles in a phase-contrast microscope or in an ordinary light microscope.

### Cytochemical Methods

Photography of the cell material near the absorption maximum of the nucleic acids at 2,570 Å was carried out in a ultraviolet microscope constructed according to Köhler, with the following optical system. Condenser: melted quartz, numerical aperture 0.6. Objective: monochromate 2.5 mm, numerical aperture 0.85. Oculars: melted quartz 5 $\times$ , 10 $\times$ . At a tube length of 16 cm and 57 cm distance to the plane of the photographic plate the linear enlargement was 1,150 times. Developer: alkaline hydroquinone for 5 minutes. All the photographs reproduced in this paper were copied on Gevaert's medium printing paper under as far as possible identical conditions.

The method elaborated by CASPERSSON (1936—1941) was used to take absorption spectra in the ultraviolet of the details of the cells. For the technical details, reference is made to CASPERSSON's original papers and to HYDÉN 1943 b, HAMBERGER and HYDÉN 1945 and HYDÉN and HARTELIUS 1948. In the quantitative data presented, principal stress is laid on comparative nucleic acid determinations in the cytoplasm of the anterior horn cells from the spinal cord in the rabbit. As has earlier been demonstrated, a small number of absorption spectra can be considered as representative of the average absorption capacity of the whole cytoplasmic mass (HYDÉN, HYDÉN and HARTELIUS). The specific absorption at 2,600 Å and at 2,750–2,850 Å was obtained after subtraction of the unspecific losses of light due to light scattering and light reflection in the cell substance. These two factors have been assumed to be equally responsible for the unspecific losses of light at 3,100 Å. In the quantitative calculations, only such curves were used that had a constant extinction value above 3,100 Å.

The absorption measurements were completed with a quantitative determination of the capacity of the cell proteins for absorbing acid dye groups at pH 2 as a measure of the amount of free basic groups (v. HYDÉN 1943). This served as a gauge of the diamino acid content in the cell structure. The method is based on an observation made by CHAPMAN, GREENBERG and SCHMIDT (1927), i. e. that certain acid dyes can react with free basic groups in the protein, which is tantamount to the sum of the free basic groups in arginine, histidine and lysine.

In stained tissue sections some of the dye groups are bound by adsorption, of which the amount increases with the duration of the staining. To eliminate this factor, the staining is carried out in the presence of a surface active agent, in this case a 0.1 per cent solution of duponol in 0.1 N HCl. The measurements for 24 hours are carried out at pH 2, where the dissociation of the  $\text{NH}_2$  groups is almost complete. The data in the following are given as a complement to earlier experiments. Erythrocytes, which are always found in every tissue section, were used as a test of uniformity in the binding of the dyes. The dye used was Cyanathrol RBX, which is bound without decolorization on the precipitation of the protein. The rabbit has, on an average, 5,359,000 erythrocytes per  $\text{mm}^3$  and 12 g of haemoglobin per 100 cc of blood. Globin is characterized by a high basic amino acid content.

An analysis of blood from a rabbit carried out according to THORNTON and ÅKESSON's micro-method (1942) gave the following results:

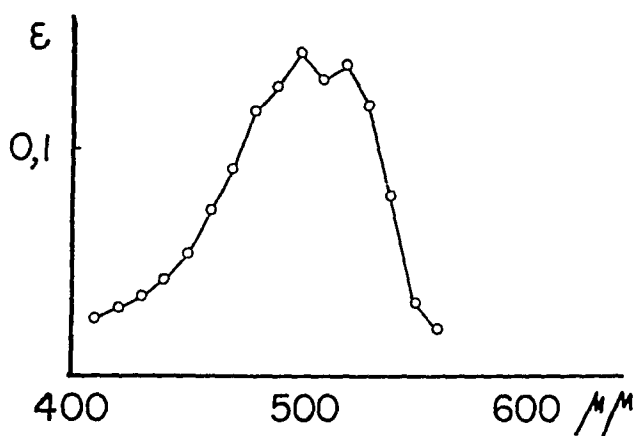


Fig. 1. Absorption spectrum of Cyananthrol RBX in water solution  
 $6 \times 10^{-7}$  g/ml.

arginine	3.33 %
histidine	8.87 %
lysine	13.51 %

Every erythrocyte contains  $2.28 \times 10^{-11}$  g of globin

of which there is  $0.123 \times 10^{-11}$  g arginine  
 $0.251 \times 10^{-11}$  g histidine  
 $0.098 \times 10^{-11}$  g lysine

It is determined from these values that each individual erythrocyte binds  $13.2 \times 10^{-12}$  g of cyananthrol RBX owing to the  $\text{NH}_2$  groups in the arginine, histidine and lysine. The other free  $\text{NH}_2$  groups in the cells protein were considered negligible.

As an example, microspectrographic determination of the amount of dye bound by a single erythrocyte is shown. The measurement was carried out at  $498_{\mu}$ , which lies at the absorption maximum of cyananthrol (fig. 1). The optical system used was an apochromatic objective, numerical aperture 0.35, compensation ocular 5 times. Enlargement in the photocell plane  $3,750 \times$ . Photocell diaphragm: 10 mm diameter, equivalent to  $2.7_{\mu}$ .

If  $\epsilon_A$  is assumed to be the extinction for 1 g of dye per 1 ml, then

$$\epsilon_{\text{erythrocyte}} = \frac{A \cdot 4 A}{d^2}$$

It is important in this calculation to know the surfaces of the erythrocytes. These were determined by a planimeter on the plates. The  $\epsilon$  indicating the shrinkage factor is determined for every section. Thus the erythrocytes were used as a test of the staining in every



Fig. 2. Anterior horn cells from the antero-lateral nucleus within the lumbar spinal cord of an untreated rabbit. Photographed at  $2,570 \text{ \AA}$ . *Optical system:* Objective 2.5 mm. Numerical aperture 0.85. Ocular:  $10\times$ . Condenser aperture: 0.6. Linear magnification  $1,150\times$ . These data apply to all the photographs in the ultraviolet range reproduced in this paper.

single case. In the experiments, the capacity of the cell substance for binding acid dye groups was determined at some important stages. The values refer to the nucleolus, the nucleus and the cytoplasm (v. p. 39).

## I. Controls

The large root cells in the antero-lateral group of the anterior horn from the lower part of the lumbar intumescences were used for the investigation. The material was thus confined to a small group of characteristic nerve cells. This group of cells in the spinal cord has been studied in earlier investigations with cytochemical methods and their composition and organization appears to be fairly constant (HYDÉN 1943 b, 1947, HYDÉN and HARTELIUS). In the last-mentioned paper, a statistical study was made of the absorption spectra taken in the ultraviolet at points in the cytoplasm of the cells representing average values for the whole cytoplasmic mass. The material was taken from three rabbits and a calculation of the distribution and amount of nucleic acids in the cells was made on the basis of the ultraviolet data. The relative changes in the protein content under various experimental conditions were also determined with the help of the absorption spectra. Since the same type of cells was taken as experimental material in the present investigation, we made use of the measurement data obtained previously as control material. A series of absorption spectra taken within various parts of an anterior horn cell, of which a photograph is seen in fig. 2, are given as examples in the following. Fig. 3 (upper curve) is taken at a point in the nucleolus and shows an absorption maximum at 2,600 Å and another strong one around 2,800 Å. Earlier studies (HYDÉN 1943 b) have shown that the proteins in the nucleolar substance, which show a strong quantitative predominance over the nucleic acid components, are characterized by a large quantity of basic groups. Absorption spectra from other points in the karyoplasm (lower curves) only show an absorption maximum at 2,800 Å, indicating protein substances only in small concentrations.

Part of the karyoplasm in the vicinity of the nucleolus nevertheless shows higher absorption in the ultraviolet photograph. This part contains protein substances of a markedly basic nature and

small concentrations of ribose nucleic acids, thus constituting a chromocentre area within the cell. This organella is extremely well developed in many of the large nerve cells and appears to constitute a part of their system which conditions the formation of the nucleoproteins in the cytoplasm. Since the present experiments also cause changes within this area of the cell, its composition and structure in the control material is emphasized.

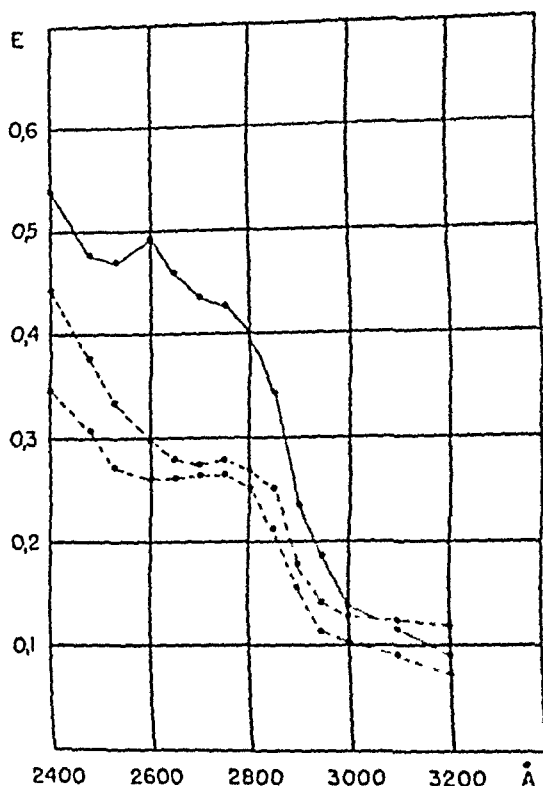


Fig. 3. Absorption spectra from the control cells reproduced in fig. 2. Upper curve from a point in the nucleolus. Dotted curves below from points in the remainder of the nucleus.

The cytoplasm of the nerve cells contains nucleic acids and protein substances in large concentrations. The curves in fig. 4 show examples of absorption spectra taken in the cytoplasm. Table IV in the quoted paper (HYDÉN and HARTELIUS 1948) gives both the original and the corrected values for the extinction at 2,600 and 2,800 Å, as well as the values obtained by graphical separation of the curves. The last column shows that the nucleic acid concentration lies, on an average, around 1.7 per cent. It can be calculated from the quotient  $E_{260}/E_{280}$



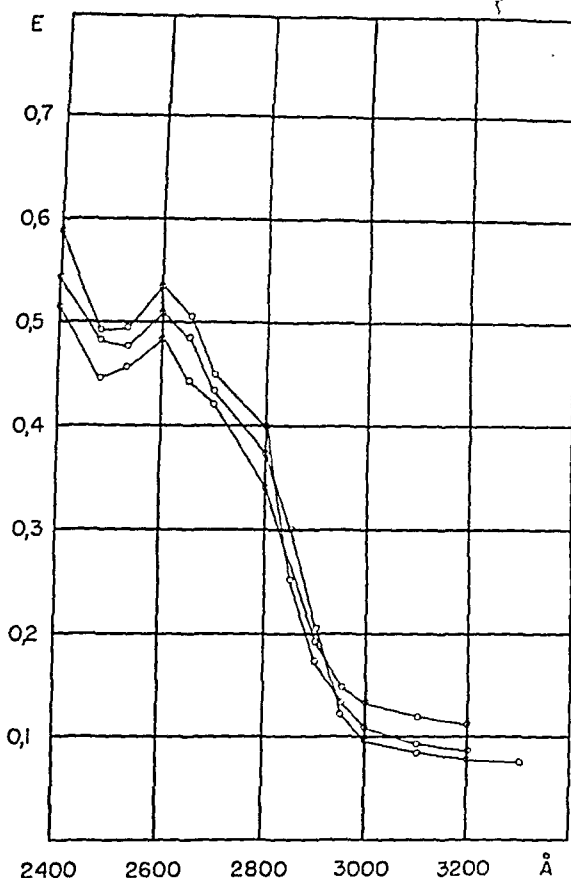


Fig. 4. Absorption spectra from points in the cytoplasm of the control cells reproduced in fig. 2.

= 1.52 that the ratio of nucleic acids to protein substances is 1:7. Referred to a "standard protein" containing 5 per cent of tyrosine and 1.5 per cent of tryptophane, the protein concentration was determined as falling within 20—30 per cent. These values are in good agreement with those obtained for the anterior horn cells using X-ray microspectrography according to ENGSTRÖM and LINDSTRÖM's method (1948).



Fig. 5. Anterior horn cell from a rabbit after occlusion of the abdominal aorta for 15 minutes. Animal killed immediately. Regarding changes of physical nature see text.

## II. Slight Spastic Paralysis After Occlusion for 15 Minutes

The specimens described as an example of this type of experiment were all taken from rabbits belonging to the same litter. After occlusion of the abdominal aorta for 15 minutes, slight spastic paralysis developed in the hind legs and remained during the survival period (v. table I). The paralysis did not develop until two and three-quarter hours after the experiment.

*It was pointed out in the section on the material that the effect of the ischaemia of the spinal cord on the motor nerve cells is a function with many variables. It was therefore impossible to assess statistically the average effect of ischaemia on the chemical composition of the nerve cells in the antero-lateral group. Analyses of individual cells are therefore given in the following as typical examples. This is also the case as regards the absorption spectra.*

### Cell changes after 1—2 hours

The animals killed one or two hours after the experiment showed no signs of paralysis or spasticity, but were extremely listless and

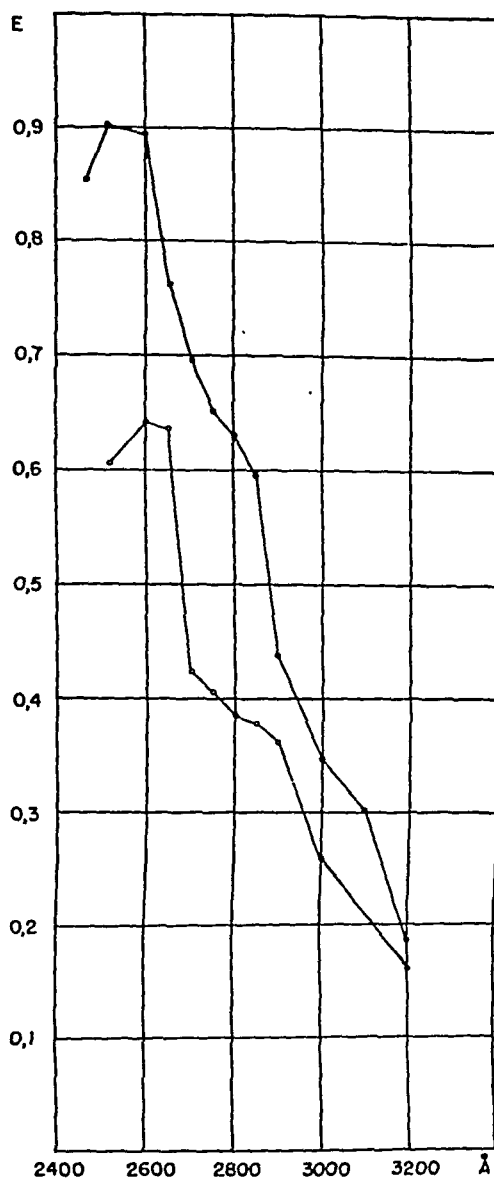


Fig. 6. Absorption spectra from a point in the nucleolus (*black circles*) and in the cytoplasm (*white circles*) of the cell reproduced in fig. 5. These designations apply to all curves reproduced in this paper.

drowsy after the anaesthetic. The material from these animals proved to be unsuitable for cytochemical investigations. The interstitial tissue of the spinal cord was very oedematous. A large area surrounding each motor cell was free from tissue. The cells were shrunk and showed a strong absorption capacity at 2,570 Å. It proved impossible to obtain acceptable absorption spectra from the majority of these cells. The unspecific losses of light were considerable owing to increased lack of homogeneity and scattering of light in the cell substance. An example of such absorption spectra is seen in fig. 6. The curves are taken at points in the cytoplasm (*the curve drawn with white circles*) and in the nucleolus from the cell in fig. 5 (*the curve with black circles*). These signs are used throughout in the present paper. Strong absorption maxima appear in both curves at 2,600 Å. The specific absorption for the cytoplasm appears particularly high. For the fore-mentioned reasons, it must be considered doubtful whether the results of the quantitative

calculations reflect the true conditions (v. diagram, fig. 13).

The oedema in the tissue had disappeared to a great extent in the animals killed three hours after the experiment. The effect of the fixation media and the histological treatment of these cells was not,

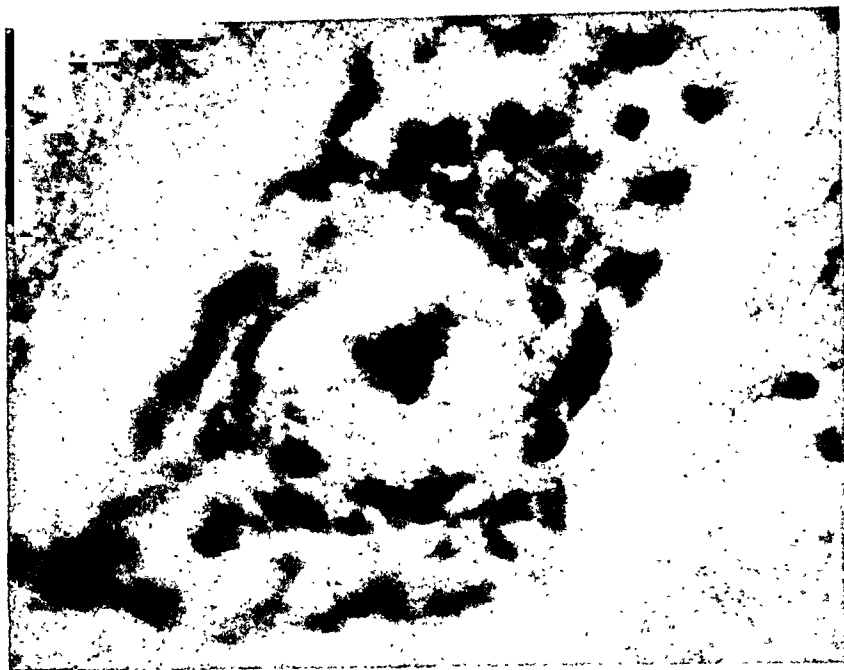


Fig. 7. Anterior horn cell from a rabbit after occlusion for 15 minutes and duration of spastic paralysis in the hind legs for 15 minutes. Observe the scanty amount of absorbing substance in the cytoplasm.

however, entirely satisfactory. The cell substance appeared fragile and was easily torn in sectioning. No shrinkage nor deformation of the cells was, however, visible in addition to the signs of changes in the physical properties of the cell substance. Since no change could be observed in the size of the unspecific losses of light as compared with the control material, the changes in the cell substance must be assumed to affect such dimensions that are not visible on optical examination in the ultraviolet range.

There were no changes of note from a technical point of view in specimens from animals killed more than three hours after the experiment.

### Cell changes after 3 hours

Moderate spastic paralysis, with a duration of 15 minutes, was present in the hind legs.

*Ultraviolet microscopy.* Fig. 7 shows a nerve cell with typical changes. The nucleolus is strongly absorbing. As regards the nuclear

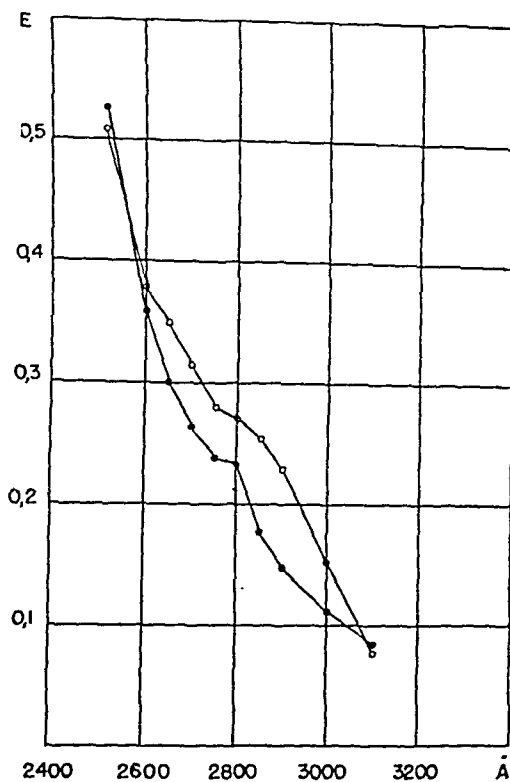


Fig. 8. Absorption spectra from points in the nucleolus and in the nucleus from the cell photographed in fig. 7.



Fig. 9. Anterior horn cell from a rabbit after occlusion for 15 minutes and a duration of spastic paralysis for 2 hours.

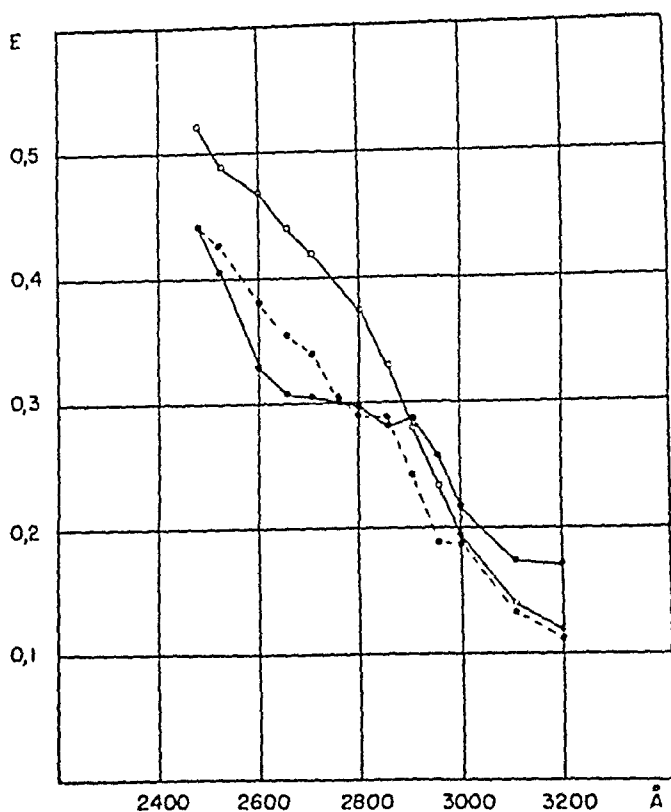


Fig. 10. Absorption of the cell photographed in fig. 9 from points in the nucleolus (black circles) in the nucleus (black circles, dotted line) and in the cytoplasm (white circles). These designations of the nucleus apply to all curves reproduced in this paper.

substance, see the foregoing. The areas within the cytoplasm which absorb strongly at  $2,570 \text{ \AA}$  are considerably scantier as compared with the control material. The greater part of the cytoplasm is characterized by a weak absorption capacity.

*Absorption spectra.* In the absorption spectra which are typical of the result of the measurements, fig. 8, only a weak absorption band appears at  $2,800 \text{ \AA}$  both for the nucleolus (the curve with black circles) and the cytoplasm. The measurements revealed the presence of protein substances only in small concentrations. No definitely measurable quantities of nucleic acids could be demonstrated from these curves.

#### Cell changes after 5 hours

The animal suffered from moderate spastic paralysis in the hind legs for two hours.

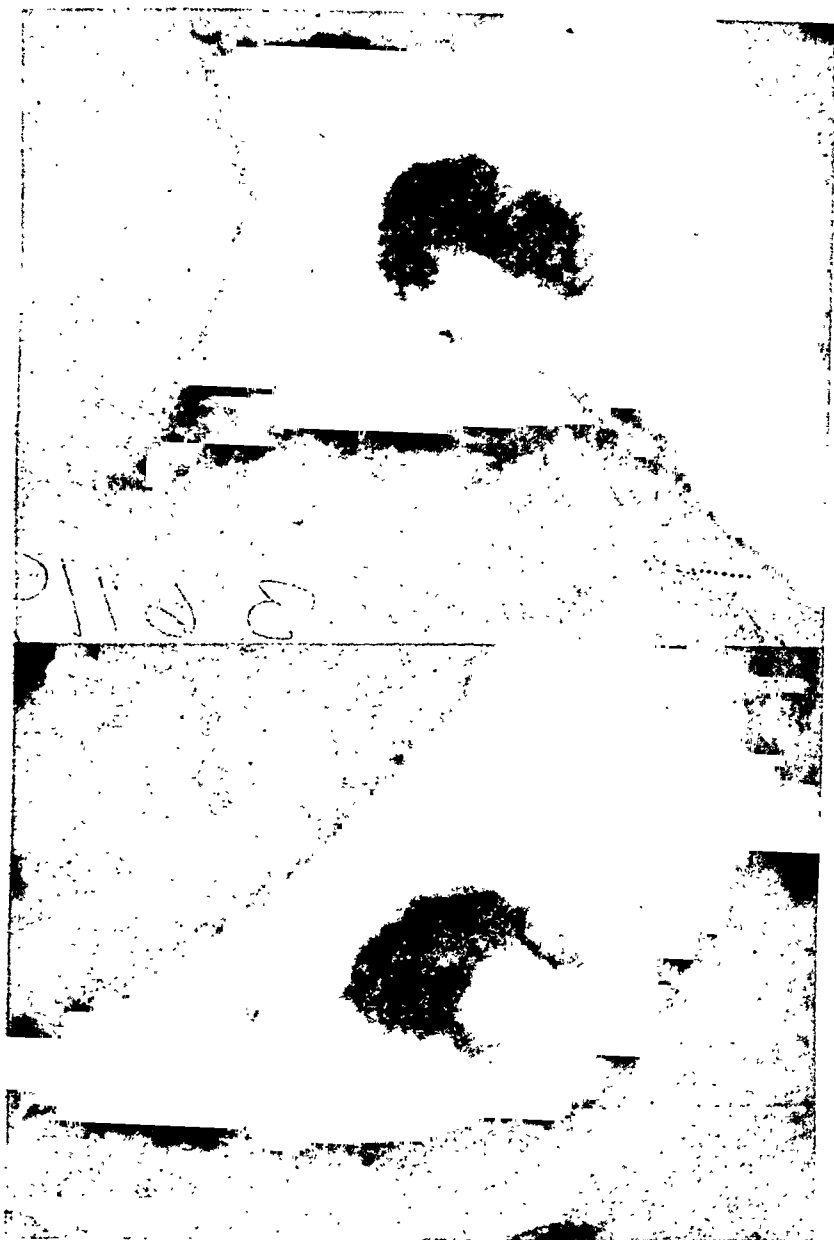


Fig. 11. Two anterior horn cells from a rabbit after occlusion for 15 minutes and a duration of spastic paralysis for  $8\frac{1}{2}$  hours. Observe the strong absorption capacity of the cytoplasm.

*Ultraviolet microscopy.* The absorption capacity is stronger in all the nerve cells than was the case three hours after occlusion. The absorption in the nuclear substance is also increased in comparison with the cells three hours after the experiment. The agglomerations

of strongly absorbing substance in the cytoplasm are larger (fig. 9) but they are fewer in number than in the control cells, in which the substance is assembled in large clumps in the cytoplasm, corresponding to the distribution of the Nissl substance. The total number of polynucleotides must therefore be substantially less than in the control cells.

*Absorption spectra.* The curves taken at points in the most intensely absorbing parts of the cytoplasm all show a weak band around  $2,600 \text{ \AA}$ , with an extinction falling evenly towards higher wave-lengths, indicating the presence of nucleic acids in small concentrations. This absorption, which increases the width of the curve, is conditioned by the band at  $2,800 \text{ \AA}$ . The absorption of the nucleolus is low around  $2,600 \text{ \AA}$  and only a weak protein band is visible at  $2,800 \text{ \AA}$ . Intrinsically the same curves are obtained from the nuclear substance as a whole (fig. 10).

### Cell changes after 12 hours

When the material was taken, spastic paralysis was present in the hind legs of the animal and had lasted for eight and a half hours.

*Ultraviolet microscopy.* All the nerve cells examined showed a

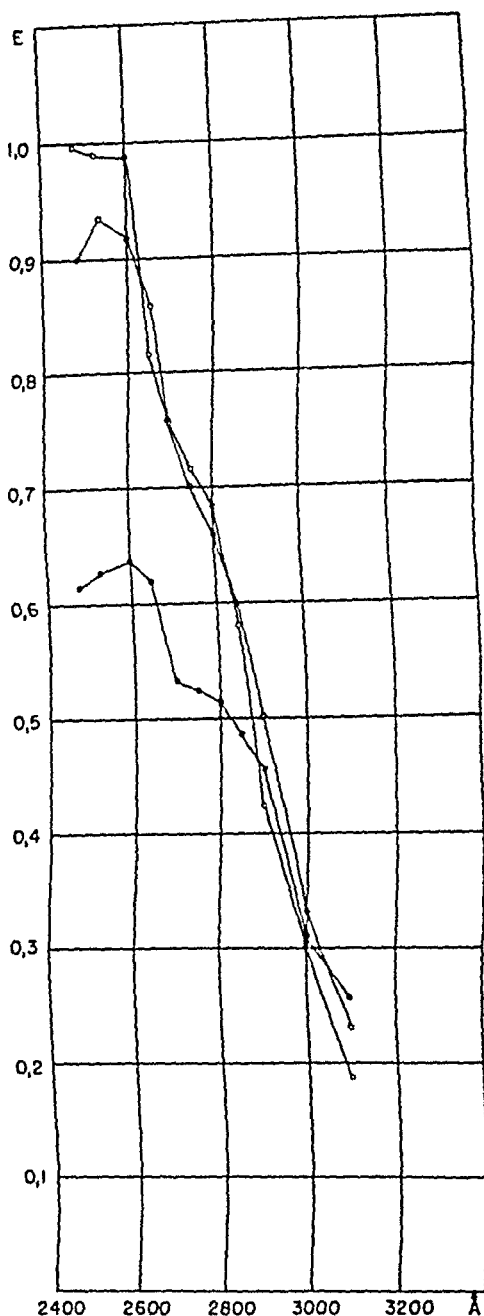


Fig. 12. Absorption spectra from points in the nucleolus and in the cytoplasm (upper two curves) of the cell photographed in fig. 11.



uniform absorption picture (fig. 11). The nucleolus absorbs strongly. The cytoplasm contains plentiful absorbing substance, diffusely spread over its whole area. The characteristic localization to certain parts within the cytoplasmic mass, corresponding to the distribution of the Nissl substance, found in the control cells is lacking here.

*Absorption spectra.* The curve in fig. 12, taken at a point in the nucleolus, shows values at 2,600 and 2,800 Å that do not differ from those obtained for nucleic acids and protein substances in the nucleoli of the control cells. Both curves taken at points in the cytoplasm show an extinction corresponding to large concentrations of nucleic acids. The absorption around 2,800 Å is also high.

### Summary

In this experimental series the abdominal aorta of altogether six rabbits was occluded bloodlessly for 15 minutes. The animals were killed at times varying between immediately after and 12 hours after the experiment. Spastic paralysis developed 2¾ hours, 3 hours, and 3½ hours respectively after the experiment, the duration varying from 15 minutes to 8½ hours. The physical condition of the cell structure in the nerve cells of animals killed immediately and up to two hours after the experiment was altered and did not permit any accurate analysis with microspectrographical methods. The cause was excessive losses of light owing to an increase in the scattering of light in the specimen.

Table IV. *Slight Spastic Paralysis After Occlusion for 15 Minutes*

Animals killed	Cell detail	E 260	E 280	E corr 260	E corr 280	E corr $\frac{260}{280}$	% Na
immediately	cytoplasm	0.642	0.387	0.379	0.170	(2.23)	(3.1)
	nucleolus	0.930	0.630	0.630	0.380	(1.66)	(3.8)
after 3 hrs.	cytoplasm	0.337	0.270	0.257	0.172	—	<0.1
Spastic paralysis for 15 mins.	nucleolus	0.357	0.231	0.234	0.128	—	<0.1
after 5 hrs.	cytoplasm	0.468	0.372	0.271	0.216	1.25	1.29
Spastic paralysis for 2 hrs.	nucleolus	0.572	0.420	0.364	0.254	1.43	2.05
after 12 hrs.	cytoplasm	0.920	0.664	0.574	0.376	1.53	4.0
Spastic paralysis for 8 hrs 30 mins	nucleolus	0.648	0.494	0.402	0.144	1.98	4.0

The development of spastic paralysis in the hind legs of the animals is accompanied by a striking decrease in the nucleoprotein content of the cytoplasm of the motor nerve cells. The decrease is extremely marked even after 15 minutes' duration. The nucleoprotein content of the nucleolar apparatus appears, on the contrary, to increase. Examples of the quantitative data illustrating the course are given in table IV.

After this period, a compensating process starts in the nerve cells, manifested as an intense activity in the nucleoprotein-producing system of the cells, causing a considerable production of cytoplasmic nucleotides. When the spastic paralysis has lasted for 8½ hours,

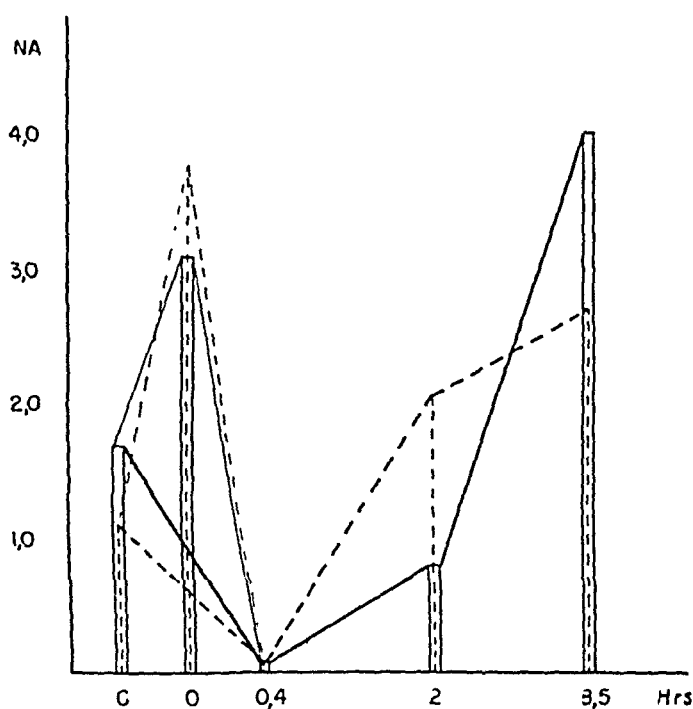


Fig. 13. Diagram showing the course of the quantitative changes in the nucleolus (dotted lines) and in the cytoplasm (unbroken lines and white columns) judged by absorption data obtained from the animals in series II. The abscissa gives the duration of the slight spastic paralysis in hours in logarithmic scale. 0 designates results from an animal after occlusion of the abdominal aorta for 15 minutes. The animal was killed immediately. The results obtained are probably due to changes in the specimen. See text. These data are therefore only indicated in the diagram. After slight spastic paralysis for 15 minutes there is a substantial decrease in the nucleoprotein content in both nucleolus and cytoplasm. After 2 hours the process is in a stage of restitution and after 8½ hours the nucleoprotein content in these parts of the nerve cell is much higher than that of the control cells.

the cytoplasm contains quantities of nucleic acids that are far in excess of those characterizing the corresponding nerve cells in the controls.

As far as it was possible to judge, there are no evident changes in the spastic paralysis after its onset. During this time, however, widespread intracellular chemical processes are taking place in the motor nerve cells. After the considerable decrease in the nucleoprotein content in the cytoplasm and inhibition of the nucleoprotein-producing system, a period follows characterized by an intense production of nucleic acids and proteins. The nucleolus plays a decisive role in this process and the results directly show the mechanism of the compensating processes during slight spastic paralysis. Compared with that of the cytoplasm, the chemical composition of the nucleolus is only very slightly affected. It is characteristic that the nucleoprotein content of the nucleolus appears to be increased during the process, indicating an increased activity in this part which is important from a point of view of the physiology of the cell (fig. 13).

### **III. Pronounced Spastic Paralysis After Occlusion for 15 Minutes**

The animals in this series also underwent ischaemia of the spinal cord for 15 minutes. In contrast to the foregoing series, this resulted in pronounced spastic paralysis in the hind legs. The animals were killed at times varying between 7 hours and 6 days after the experiment (table II). As regards time, this series partly overlaps the preceding one, but in addition permits a study of the cell changes at a longer time after the onset of paralysis. In two cases it was thus possible to investigate the marked spasticity in the hind legs. These two experiments resulted in the condition termed "irritative state" with muscular hypertonus by earlier writers.

#### **Cell changes after 4 hours**

Spastic paralysis, which had lasted for two hours, was still present four hours after the experiment.

*Ultraviolet microscopy.* As a whole, the cells absorb poorly at 2,570 Å. The large nucleolus, however, is clearly visible, owing to

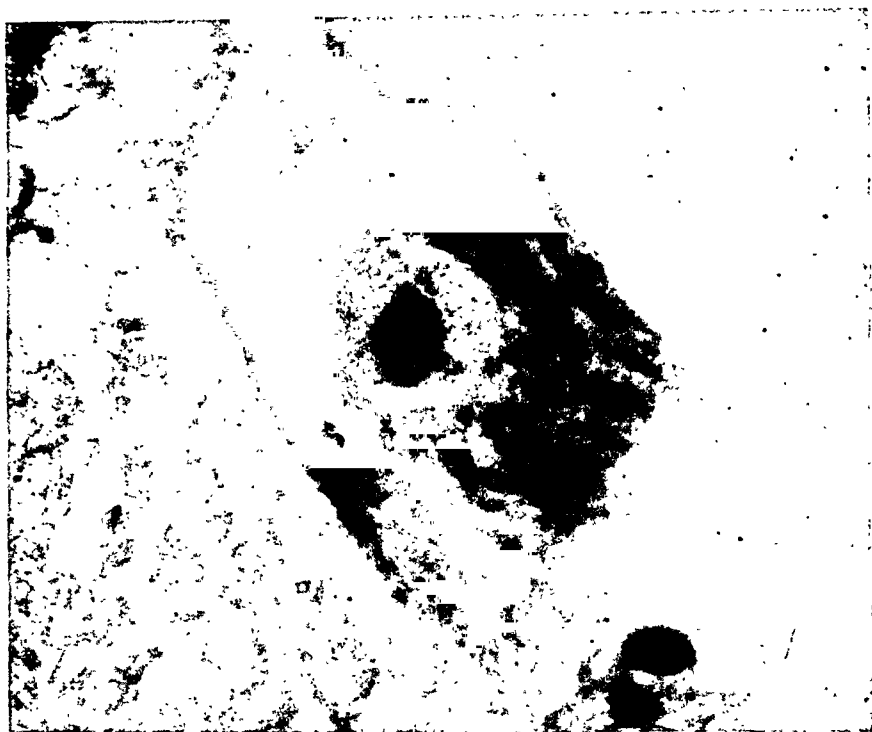


Fig. 14. Anterior horn cell from a rabbit after occlusion for 15 minutes and pronounced spastic paralysis for 2 hours. Survival time 4 hours. The nucleolus absorbs at  $2,570 \text{ \AA}$  but the absorption capacity of the cytoplasm is poor.

its more intense absorption (fig. 14). Only sparse absorbing areas are seen in the cytoplasm.

*Absorption spectra.* The curves (fig. 15) show, in respect of the nucleus, an absorption maximum at  $2,600 \text{ \AA}$ , indicating the presence of a moderate nucleic acid content. Absorption spectra taken at points in the remainder of the nucleus have an absorption band at  $2,800 \text{ \AA}$ . The example of a curve from the cytoplasm has only very slight such absorption, showing the presence of proteins in small concentrations.

#### Cell changes after 7 hours

The animal suffered from moderately pronounced spastic paralysis during 30 minutes.

*Ultraviolet microscopy.* The cells as a whole are characterized by a less intense absorption capacity as compared with those of the controls (v. fig. 16). There is, however, an exception in the nuclear

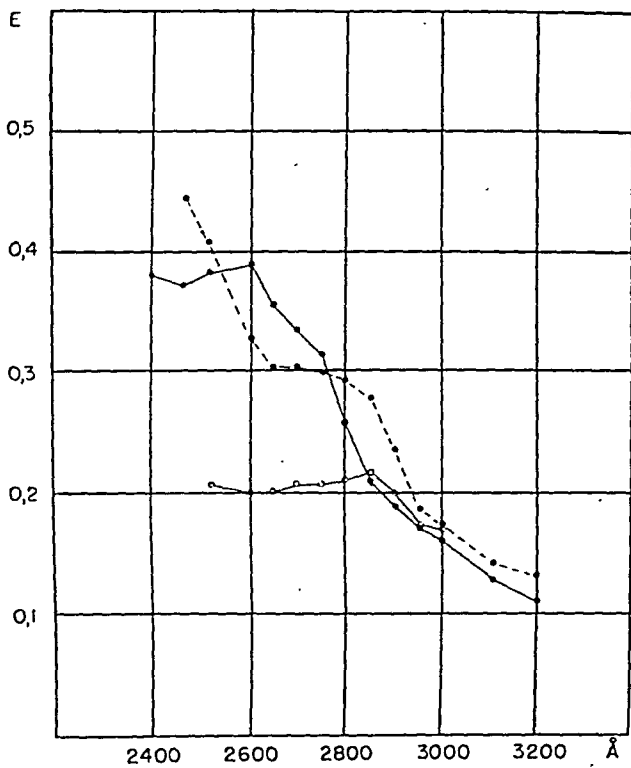


Fig. 15. Absorption spectra from points in the nucleolus, nucleus and cytoplasm of the cell photographed in fig. 14. Note the faint absorption at 2,800 Å from the cytoplasm.



Fig. 16. Anterior horn cell from a rabbit after occlusion for 15 minutes and pronounced spastic paralysis for 30 minutes. Survival time 7 hours. Note the slight absorption capacity of the cytoplasm compared with that of the controls. In the cytoplasm only small absorbing areas are visible. Observe the clearly visible chromocentre areas in the vicinity of the nucleolus.

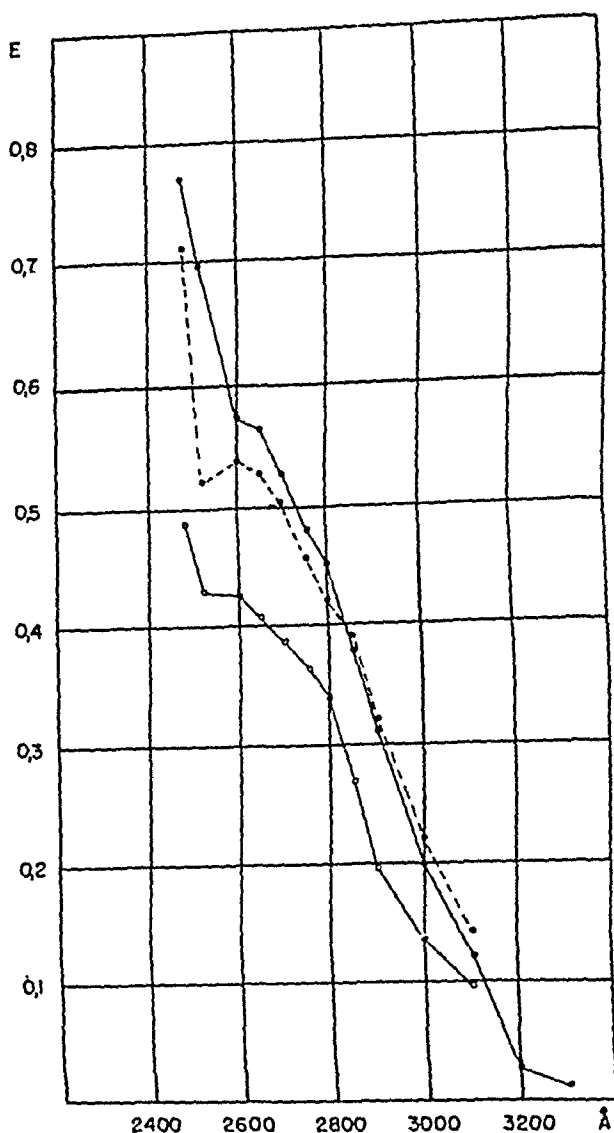


Fig. 17. Absorption spectra from points in the nucleolus, nucleus and cytoplasm of the cell photographed in fig. 16.

substance. As described on p. 16, there is a well developed chromocentre area in the large motor root cells of the controls. Seven hours after occlusion of the abdominal aorta, these areas are enlarged and very distinct, making the absorption capacity of the nucleus stronger. In the nerve cells in this experimental series, there are very few such strongly absorbing areas.

*Absorption spectra.* The courses of the curves in fig. 17, taken at points in the cytoplasm and in the nuclear substance in its vicinity,

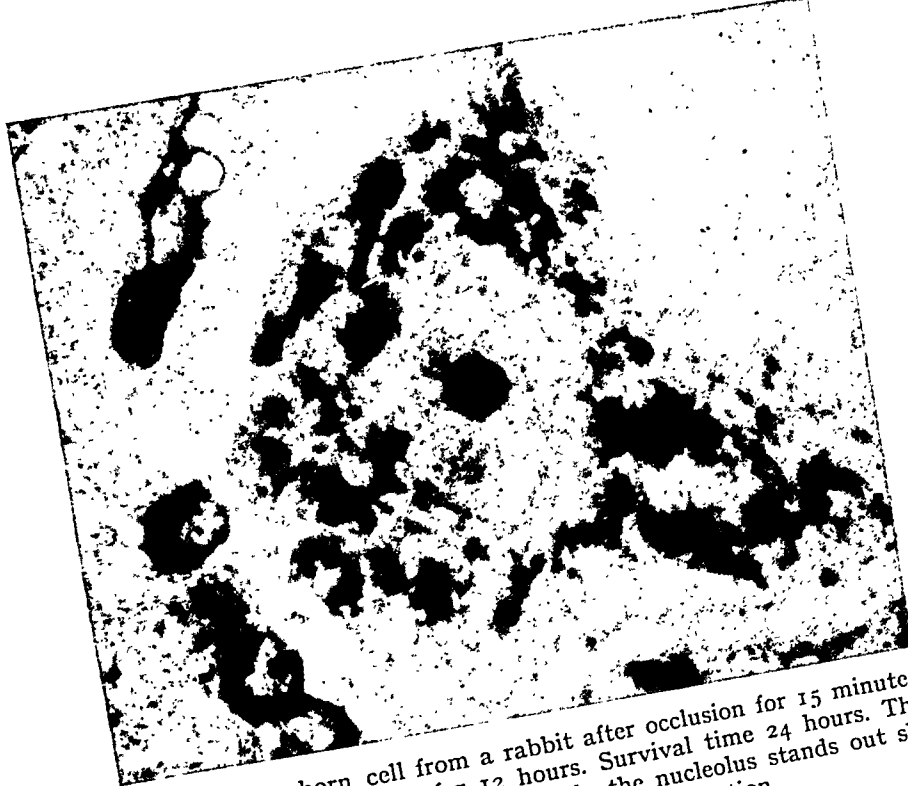


Fig. 18. Anterior horn cell from a rabbit after occlusion for 15 minutes and pronounced spastic paralysis for 12 hours. Survival time 24 hours. The cell substance absorbs poorly at 2,570 Å. Only the nucleolus stands out sharply and is characterized by a high absorption.

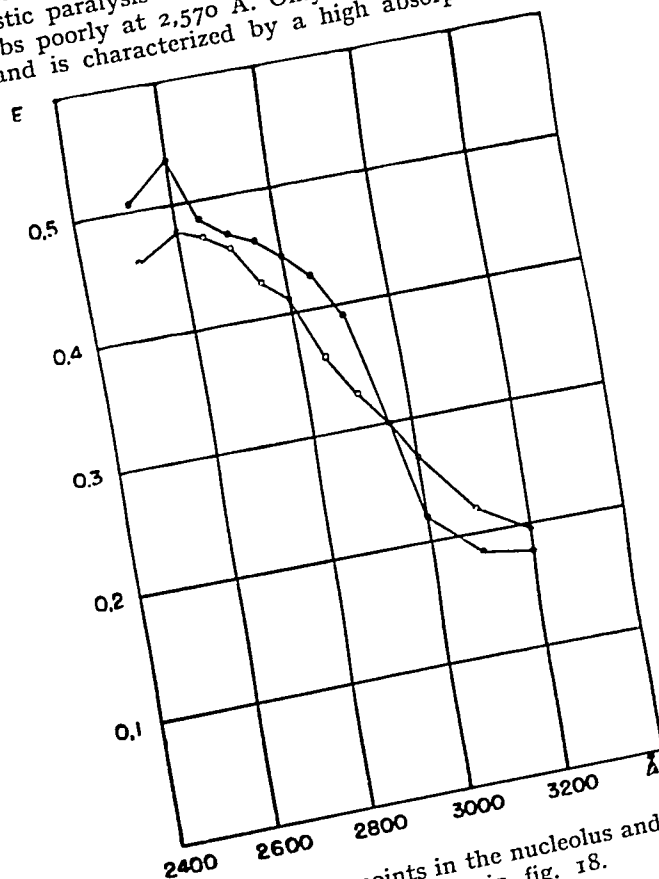


Fig. 19. Absorption spectra from points in the nucleolus and in the cytoplasm of the cell photographed in fig. 18.

are in good agreement. Both curves show a distinct maximum at  $2,600 \text{ \AA}$  and the absorption around  $2,800 \text{ \AA}$  is also considerable, giving increased breadth to the curves. In the nucleolar substance, the specific absorption at  $2,600 \text{ \AA}$  is greater, indicating a nucleic acid content. The absorption curves from the cytoplasm also show the same intrinsic — although less pronounced — nucleic acid band, corresponding to smaller but definitely measurable quantities of nucleic acids.

### Cell changes after 24 hours

Spastic paralysis occurred in the hind legs of this animal 12 hours after occlusion and was still present when the animal was killed.

*Ultraviolet microscopy.* Fig. 18 shows a typical example of the anterior horn cells in this animal. On the whole, the cell absorbs poorly. The nucleolus is nevertheless strongly absorbing and its structure is well defined.

*Absorption spectra.* The curve in fig. 19, taken at a point in the cytoplasm, shows the presence of nucleic acids. It also has a marked protein absorption. Nucleic acids in small concentrations are shown by the curves from the cytoplasm.

### Cell changes after 72 hours

After occlusion of the abdominal aorta, spastic paralysis, persisting for 24 hours, developed. The animal was killed after a further 12 hours. The paralysis had then regressed, but there was definite *spasticity* in the musculature of the hind legs.

*Ultraviolet microscopy.* The nerve cells in fig. 20 show a strong absorption capacity at  $2,570 \text{ \AA}$ . Their appearance is characteristic and it is thus easy to distinguish them in the ultraviolet photograph from the control cells. The absorbing substance is localized to small areas, giving the cytoplasm a "granulated" appearance. The agglomerations are not sharply defined in most cases and the cells therefore appear blurred. Their appearance in the ultraviolet appears to be characteristic of nerve cells during spasticity. See further p. 38.

*Absorption spectra.* The curves taken in the nucleolus, fig. 21, only show a weak maximum around  $2,600 \text{ \AA}$  and at  $2,800 \text{ \AA}$ . The remainder of the nuclear substance has, in principle, the same absorption spectra with the same course, although the extinction values are lower. The curves taken in the cytoplasm indicate the presence of a high content of nucleic acids and proteins.





Fig. 20. Anterior horn cells from a rabbit after occlusion for 15 minutes and pronounced spastic paralysis for 24 hours followed by remaining muscular hypertonus in the hind legs for 12 hours. Survival period 72 hours. The cytoplasm is rich in absorbing substance localized to small areas, which in the upper cell have no distinct boundaries, giving the cytoplasm a "blurred" appearance.

## Cell changes after 96 hours

At death, this animal had pronounced spastic paralysis, which had lasted for altogether 50 hours. No difference was found in the material at first sight as regards the motility or the muscular tonus of the hind leg on either side. Cytochemical analysis nevertheless revealed a distinct difference between the anterior horn cells on the left and on the right side. The findings in the anterior horn cells in the left part of the spinal cord are described first in the following.

### a. Left side

#### *Ultraviolet microscopy.*

The cell in fig. 22 is characterized by intense absorption in the ultraviolet. The absorbing substances lie in well defined areas in the cytoplasm.

*Absorption spectra* from the nucleolus show the presence of considerable concentrations of nucleic acids and protein substances (v. fig. 23). Absorption measurements in the cytoplasm reveal nucleic acids in these cells in the same quantities as in the control material. The absorption band at  $2,600 \text{ \AA}$  is particularly strong and this is also the case as regards the specific absorption at  $2,800 \text{ \AA}$ .

### b. Right side

The cells from the right side gave the same results as those obtained for the nerve cells following spastic paralysis of short duration (v. p. 21 and table I).

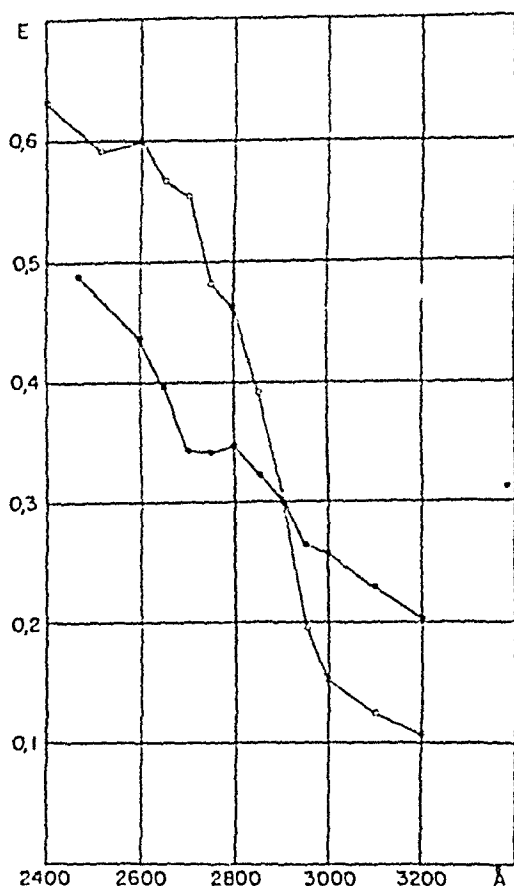


Fig. 21. Absorption spectra from points in the nucleolus and the cytoplasm of the cells photographed in fig. 20.

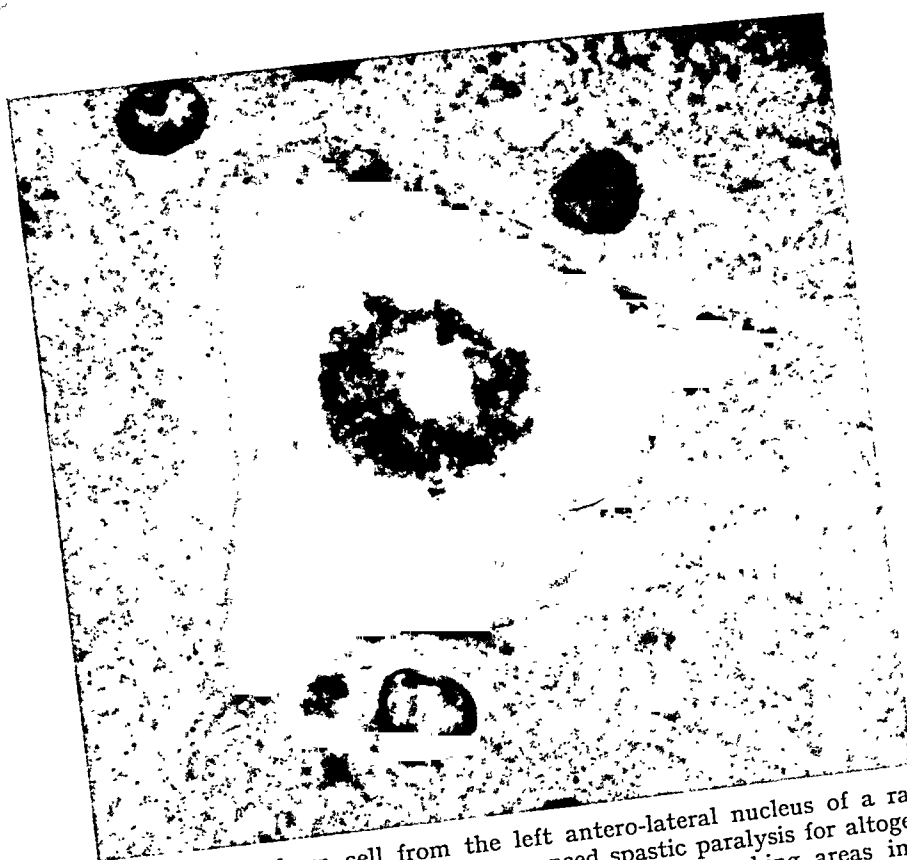


Fig. 22. Anterior horn cell from the left antero-lateral nucleus of a rabbit after occlusion for 15 minutes and pronounced spastic paralysis for altogether 50 hours. Survival time 96 hours. Well developed absorbing areas in the cytoplasm.

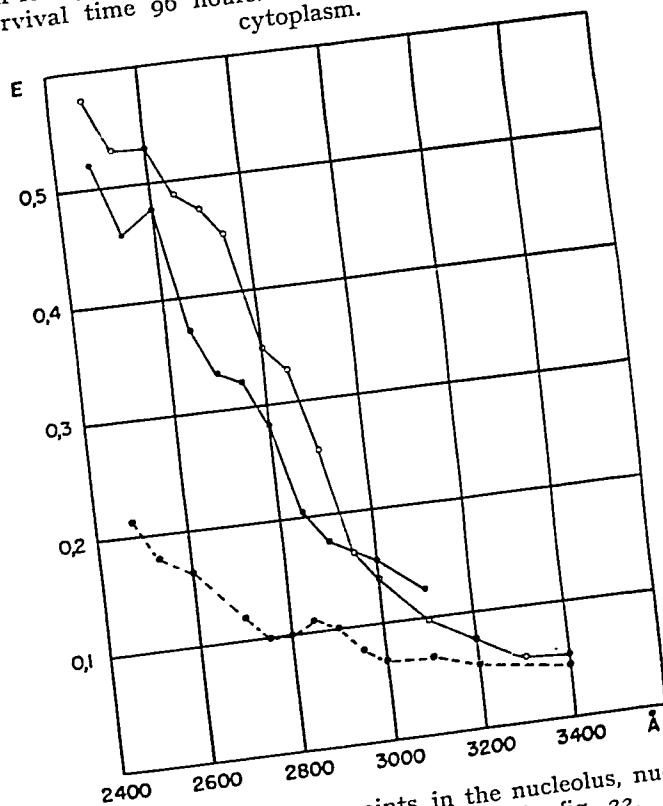


Fig. 23. Absorption spectra from points in the nucleolus, nucleus and cytoplasm of the cell photographed in fig. 22.

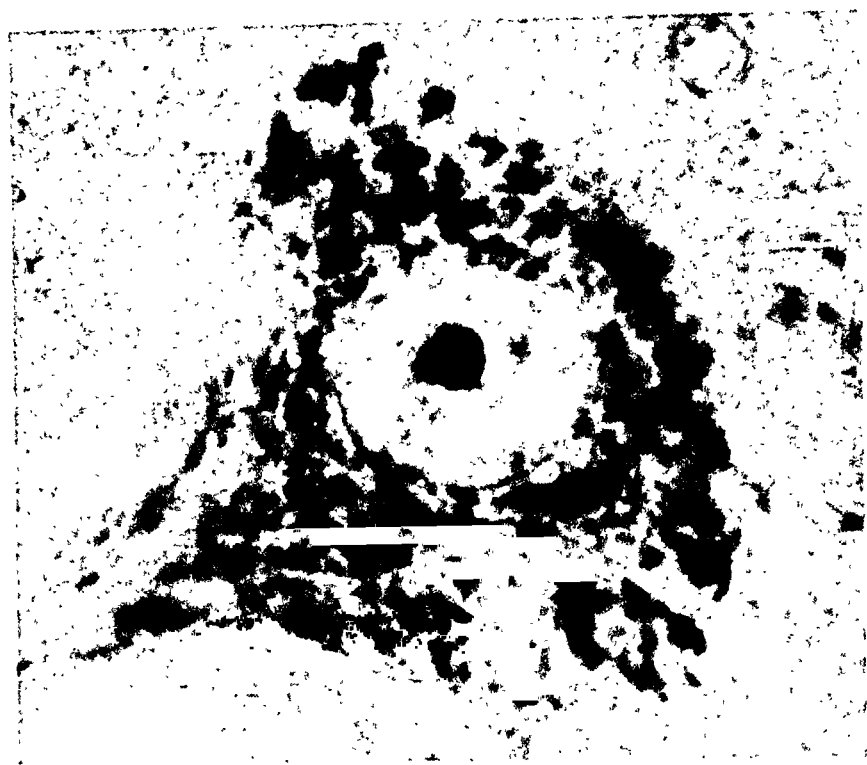


Fig. 23 b. Anterior horn cell from the right antero-lateral nucleus of a rabbit after occlusion for 15 minutes and pronounced spastic paralysis for altogether 50 hours. Survival time 90 hours. The cell absorbs poorly at  $2,570 \text{ \AA}$  compared with corresponding cells from the left side.

*Ultraviolet microscopy.* Strongly absorbing substance at  $2,600 \text{ \AA}$  is lacking (v. fig. 23 b). The nerve cells are characterized by a scanty absorption capacity. Only the nucleolus absorbs intensely and its structure is well defined.

*Absorption spectra.* The curves taken in the nucleolus indicate the presence of sparse concentrations of nucleic acids and proteins. Absorption spectra from points in the nucleolus reveal no measurable quantities of nucleic acids and only small concentrations of proteins.

#### Cell changes after 6 days

As a last step in these experiments, material from a rabbit that had survived six days after the occlusion of the abdominal aorta was studied. During the first 48 hours spastic paralysis occurred in the hind legs. During the remainder of the time this was replaced by an increased spasticity, which remained until the death of the

animal. As in the foregoing case, we obtained a distinct difference between the right and the left side in respect of the nucleoprotein content of the anterior horn cells. Strongly absorbing cells, containing considerable quantities of nucleoproteins both in the nucleolus and in the cytoplasm, were found on the left side. Ultraviolet microscopy revealed the same appearance in the cytoplasm as that described in an animal after 72 hours (v. p. 33). Analysis of the spinal cord gave results in agreement with those obtained in the foregoing experiment, in which the material was taken 96 hours after occlusion. In the right side of the spinal cord the chemical composition of the anterior horn cells was the same as that in the corresponding material in the foregoing experiment. Both the nucleolus and the cytoplasm were characterized by a low nucleoprotein content.

*Examples of Quantitative Data Obtained From the Motor Nerve Cells After Occlusion of the Abdominal Aorta for 15 Minutes*

*Table V. Pronounced Spastic Paralysis After Occlusion for 15 Minutes*

Animals killed	Cell detail	E 260	E 280	E corr 260	E corr 280	E corr $\frac{260}{280}$	% Na
after 4 hrs. Spastic paralysis for 2 hrs.	cytoplasm nucleolus	— 0.388	— 0.256	— 0.218	— 0.126	— 1.73	— 1.39
after 7 hrs. Spastic paralysis for 30 mins.	cytoplasm nucleolus	0.424 0.570	0.336 0.445	0.280 0.530	0.223 0.416	1.23 1.27	0.46 1.34
after 24 hrs. Spastic paralysis for 12 hrs. At death spasticity	cytoplasm nucleolus	0.480 0.536	0.412 0.447	0.151 0.241	0.142 0.204	1.07 1.18	0.57 1.40
after 72 hrs.	cytoplasm nucleolus	0.600 0.436	0.460 0.345	0.413 0.106	0.307 0.075	1.35 1.41	2.21 1.0
after 96 hrs. Spastic paralysis for 50 hrs.	cytoplasm nucleolus	0.530 0.478	0.350 0.280	0.414 0.295	0.255 0.218	1.63 2.30	2.64 2.1

The uncorrected and the corrected values for the extinction at 2,600 Å and 2,800 Å, the quotient of these values and the nucleoprotein content, calculated on the basis of these figures, are given in the table.

# *The Capacity of the Nerve Cells for Binding Acid Dye Groups*

Table VI

Animals	Free space	Cyto- plasm	Nucleus	Nucleolus
Controls.....	0.0	0.171	0.09	0.21
Spastic paralysis for 30 mins..	0.0	0.155	0.069	0.184
Spastic paralysis for 50 hrs. Mean value of 4 series of measurements	0.0	0.037	0.102	0.138
Spastic paralysis for 6 days. Mean value of 5 series of measure- ments.....	0.0	0.099	0.108	0.325

In order to obtain information regarding the amount of basic groups in the cell protein, measurements of its capacity for binding dyes were made according to the method described on p. 13. These measurements were only made in a small number of instances, in which the microspectrographical measurements in the ultraviolet range revealed substantial changes.

Mean figures are given as typical examples in table VI. A decrease in the nucleoprotein content of the cytoplasm could be demonstrated after paralysis with a duration of 30 minutes (table V). The extinction on measurement of the capacity for binding acid dyes does not, however, differ greatly from the figures for the controls.

As typical examples, values are given in table VI for cells with a low nucleotide content from animals with spastic paralysis for 50 hours. The table shows that the nucleolar substance contains a small quantity of basic groups. This is also the case for the cytoplasm. This finding supports the conclusions made on the basis of the absorption data, i. e. that the nucleoprotein is inhibited in this type of cell.

After paralysis with a duration of six days, the nucleoprotein content of the nerve cells was higher than in the last-mentioned type of cell. The cytoplasmic substance contained a relatively small quantity of basic groups compared with the control cells. The nucleolar substance, on the contrary, had a large amount of basic groups. The result thus confirms the conclusion made on the grounds of the absorption data, namely that the nucleolus in these cells is intensely active.

## Diagram of Quantitative Changes

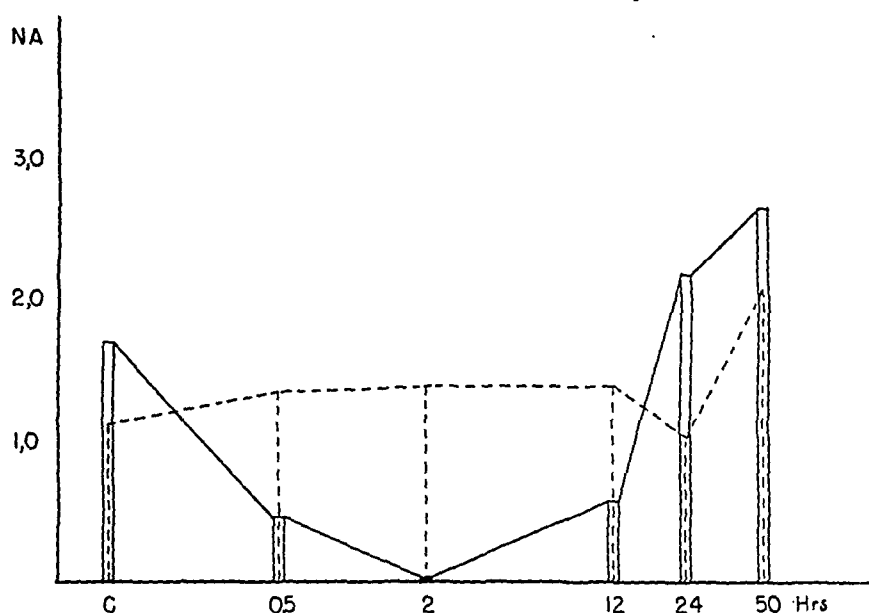


Fig. 24. Diagram showing the course of the quantitative changes in the nucleolus (*dotted lines*) and in the cytoplasm (*unbroken lines and white columns*) judged by absorption data obtained from the animals listed in table II. The abscissa gives the duration of spastic paralysis in hours in logarithmic scale. After a duration of the pronounced spastic paralysis for 0.5, 2 and 12 hours there is a substantial decrease in the nucleoprotein content in the cytoplasm, the decrease being most marked after 2 hours. After a duration of the paralysis for 12 and 50 hours the nucleoprotein content has exceeded that of the control cells, indicating a stage of lively restitution.

During the course of this process the nucleoprotein content of the nucleolus is maintained.

## Survey of Results, II, III

In the experimental series described in the foregoing, the occlusion of the abdominal aorta lasted for 15 minutes. Its effect was manifest functionally as a spastic paralysis, as in the experiments described under I (v. pp. 19—28). The experiments were characterized by the reversibility of the functional lesions in some cases, and even the chemical changes showed signs of regression. The experiments described cover various stages in this process. It was therefore possible to make a correlation between the function of the neurons and the intracellular chemical changes in corresponding motor nerve cells.

The two experimental series with occlusion of the abdominal aorta for 15 minutes nevertheless differ in that the extent of the damage varied. In the series summarized in table I and diagram in fig.

13, only slight spastic paralysis developed in the hind legs. In the series in table II the paralysis was of the same type although more pronounced. This experiment differs from the foregoing by means of its more lengthy course.

The animals were killed immediately or up to six days after the experiment. Both experimental series overlap as regards the 5—12 hour period after occlusion. The onset of paralysis varied between two hours to 12 hours after the occlusion. Its longest duration was 48 hours and the shortest 30 minutes.

The chemical composition of the nerve cells in respect of their nucleic acid and protein content and the nuclear apparatus was studied: 1. at the onset of paralysis, 2. at varying durations of spastic paralysis up to 48 hours, 3. in residual muscular hypertonus in the hind legs without true paralysis, 4. after initial restitution.

Distinct spastic paralysis could be observed two hours after the end of the experiment.

When the paralysis had lasted for 30 minutes, a substantial decrease could be noted in the nucleic acid and protein content of the cytoplasm in the motor cells involved. This condition can be interpreted as a temporary inhibition of the nucleoprotein-producing system of the nerve cell, with the changes in the nucleolar apparatus as its most important manifestation. The chemical changes can be correlated with the clinical symptoms, i. e. spastic paralysis.

A process of restitution nevertheless appears to start immediately, an indication being the increase in the nucleoprotein content of the cytoplasm. The nucleoproteins in the nerve cells in this restorative phase are diffusely spread throughout the cytoplasm. They differ in this respect in the ultraviolet photograph from the cells of the controls, in which the nucleoproteins are present in well defined areas. Such pictures of restitution are found after a duration of the spastic paralysis of both 12 and 24 hours.

The diagram in fig. 24 depicts graphically the chemical process in the nerve cells during pronounced spastic paralysis following occlusion of the abdominal aorta for 15 minutes. It is seen that, as in the foregoing experimental series, the reaction of the nucleolus is considerably less extensive than that of the cytoplasm. The former retains its original content of ribose nucleic acids or even increases its proportion of these substances during the phase when the cytoplasm shows an almost complete lack of nucleoproteins.



*This chemical composition in the nucleolus --- which is an important directive centre for the production of the cytoplasmic nucleoproteins --- appears to be necessary in order that possibilities may exist for restitution and for maintenance of the function of the nerve cell.*

The experiment also permitted a study of the cytochemical changes in the nerve cells under conditions of *spasticity* or *muscular hyper-tonus*. These conditions appear to have a characteristic chemical correlate in the motor nerve cells in the area involved.

In the forementioned conditions, the nerve cells have large quantities of nucleic acids and proteins in their cytoplasm. Changes of a physical nature appear to have taken place in the cell substance, altering the aggregation condition of the parts rich in nucleic acids. The substance with a high nucleic acid content is found in the form of small, indistinctly defined agglomerations, giving a very characteristic appearance to the cell.

With the methods used here, no changes could be found in the cytochemical composition as compared with the control cells in those cases in which functional restitution had started.

#### **IV. Severe Spastic Paralysis After Occlusion for 25 Minutes**

In these experiments the animal manifested severe spastic paralysis in the hind legs as well as incontinentia vesicae et alvi. The paralysis could be noted during the first hours after the termination of anaesthesia. As regards its type and course, it was entirely in conformity with the results obtained by HÄGGQVIST (1937, 1938) and confirmed and elaborated by REXED (1940) using the same technique. After occlusion for 30 minutes and with a survival time of up to three weeks, HÄGGQVIST noted that the large motor anterior horn cells in the spinal cord were the first to be affected. Three weeks after the experiment these cells were found to be entirely destroyed. He found a simultaneous disappearance of the coarse neurites in the anterior roots. It is seen from table III that in the present experiments the large root cells in the spinal cord within the antero-lateral nucleus were studied at times varying from 8 hours to one month after such an experiment. The changes are thus irreversible in such cases and destruction of the cells takes place in a short time.

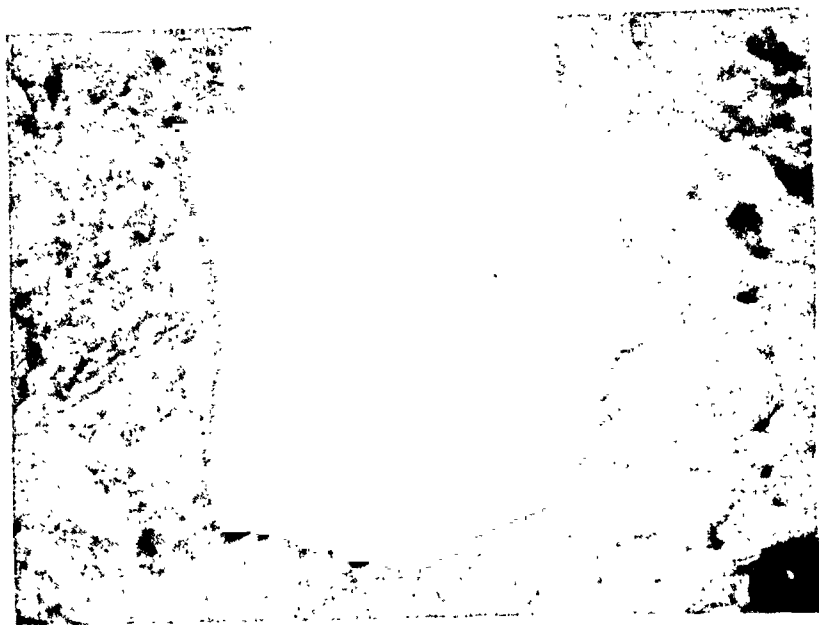


Fig. 25. Anterior horn cell from a rabbit after occlusion for 25 minutes and severe spastic paralysis for approximately 8 hours. The cell as a whole is characterized by a moderate but diffuse absorption at  $2,570 \text{ \AA}$ .

### Cell changes after 8 hours

Severe spastic paralysis developed at the end of the experiment. Two types of cell changes were encountered in the nerve cells in the antero-lateral nucleus. The first was characterized by a weak absorption capacity as compared to the control cells. The second was characterized by an extremely poor absorption capacity with the exception of the nuclear substance which was, however, shrunken.

Examples of analyses of both types of cells are given in the following.

*Ultraviolet microscopy.* Fig. 25 shows a nerve cell which is characterized as a whole by a decrease in the absorption capacity at  $2,570 \text{ \AA}$  compared with corresponding cells from the controls. That of the nucleus is striking when compared to the intense absorption in the control cells. The moderately absorbing substance in the cytoplasm is localized to small areas with a somewhat more intense absorption capacity, giving the cell mass a finely-granulated appearance.

*Absorption spectra.* The curve taken in the nucleolus of the cell in fig. 25 shows an absorption maximum at  $2,750 \text{ \AA}$  and a minimum at  $2,600 \text{ \AA}$  (fig. 26). The nucleolar substance thus contains only

protein substances. The curve from the cytoplasm indicates the presence of small concentrations of nucleic acids.

The second type of cell is demonstrated by the photograph in fig. 27.

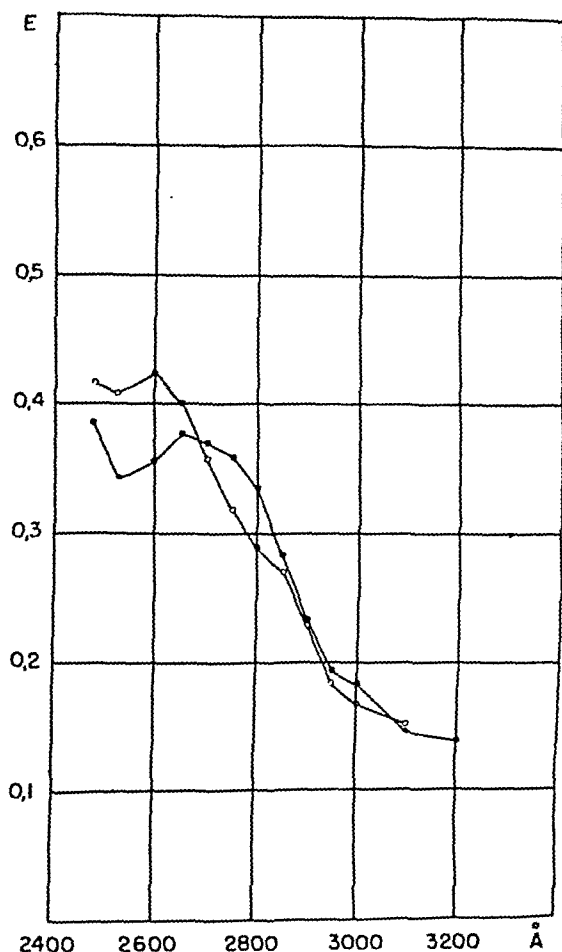


Fig. 26. Absorption spectra from points in the nucleolus and in the cytoplasm of the cell photographed in fig. 25.

#### *Ultraviolet microscopy.*

The nuclear substance is shrunken. The nucleolus nevertheless stands out by reason of its strong absorption and differs neither as regards shape nor structure from the nucleoli of the control cells. The absorption capacity of the cytoplasm is particularly poor at 2,600 Å. Streaks of more intensely absorbing substance are seen in the cytoplasmic mass, giving it a sparsely striated appearance.

#### *Absorption spectra.*

The curve taken at a point in the nucleolus (fig. 28) shows a strong band at 2,600 Å. Owing to the deformation seen in the nuclear substance, it was not considered justified to draw any quantitative conclusions from curves from this type of cell.

The example of a curve in the cytoplasm only shows the presence of very small concentrations of protein substances.

### **Cell changes after 48 hours**

The animal had severe spastic paralysis in the hind legs and paresis in the sphincter muscles of the bladder and intestine.

*Ultraviolet microscopy.* The nerve cells from this animal contain absorbing substance at 2,570 Å, localized to small areas in the



Fig. 27. Anterior horn cell from a rabbit after occlusion for 25 minutes and severe spastic paralysis for approximately 8 hours. The nucleus is shrunken and no distinct limitation of the nucleolus is visible. The cytoplasm is characterized by a very faint absorption capacity at 2,570 Å.

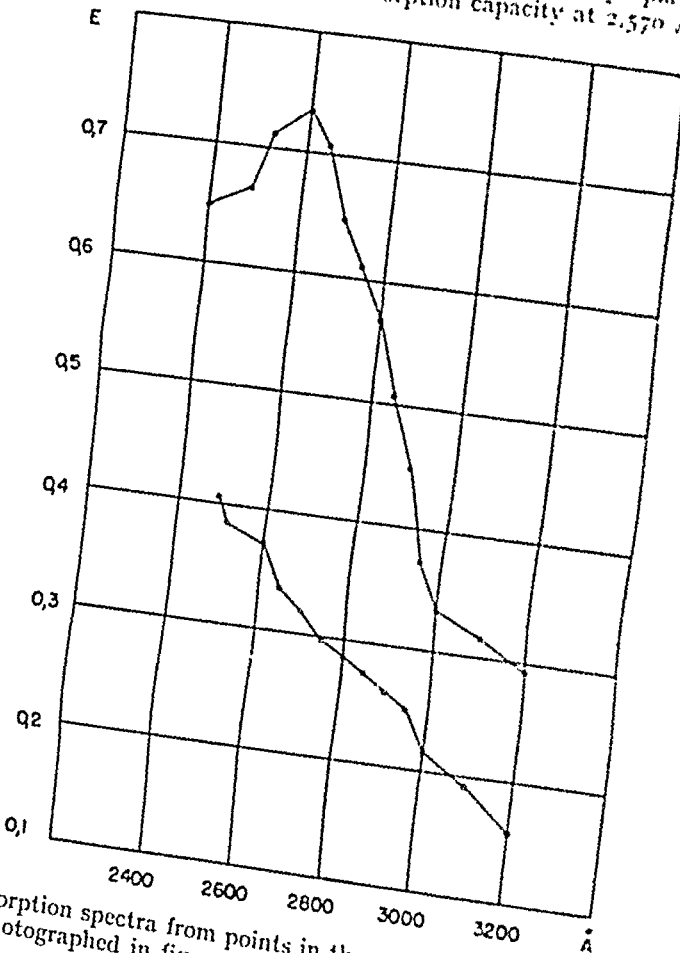


Fig. 28. Absorption spectra from points in the nucleolus and in the cytoplasm of the cell photographed in fig. 27. Regarding the interpretation of the curve from the nucleolus, see text.



Fig. 29. Anterior horn cell from a rabbit after occlusion for 25 minutes and severe spastic paralysis for approximately 48 hours. In the cytoplasm small areas of moderate absorbing substance giving a fine granulated appearance to the cell substance.

cytoplasm, whose borderlines are not sharply defined against the surrounding substance (fig. 29). It is particularly noticeable that the chromocentre areas in the nucleus are markedly enlarged and stand out through their intense absorption. This was evident in all the nerve cells from this animal. The findings are such that they will be discussed in detail in a special section (v. p. 55).

*Absorption spectra.* The nucleolus from this type of cell gives an absorption curve with a pronounced maximum around  $2,600 \text{ \AA}$  and at  $2,800 \text{ \AA}$ , showing the presence of considerable concentrations of nucleoproteins (v. fig. 30). The remainder of the nuclear substance, with chromocentre areas occupying practically the entire nucleus, gives an absorption spectrum showing the presence of small concentrations of nucleic acids and considerable amounts of proteins. Curves from points in the cytoplasm show the presence of nucleic acids in smaller concentrations than in the nucleolus.

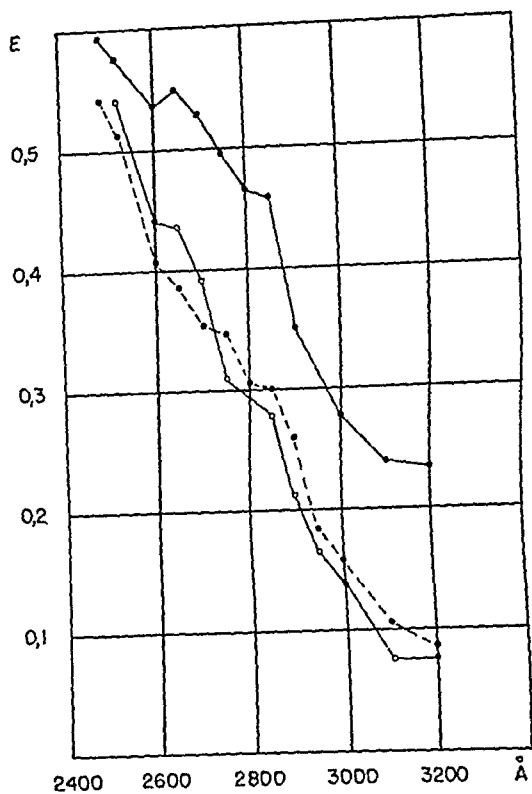


Fig. 30. Absorption spectra from points in the nucleolus, nucleus and the cytoplasm of the cell in fig. 30.



Fig. 31. Anterior horn cell from a rabbit after occlusion for 25 minutes and severe spastic paralysis for approximately 6 days. The cell is intensely absorbing at 2,570 Å. In the cytoplasm the structure seems vacuolized.

### Cell changes after 6 days

The animal, as did the others, had severe spastic paralysis in the hind legs and paresis of the sphincter muscles of the bladder and intestine. On examination

of survey preparations from the area in the spinal cord involved, cell remains from neuronophagocytosed cells were seen at the site of the anterior horn as well as a few cells of the appearance seen in fig. 31.

*Ultraviolet microscopy.* In the ultraviolet photograph the cell is seen to be intensely absorbing. Closer study of the photographs reveals vacuolization of the cytoplasmic structure. The nuclear substance appears to be considerably shrunken.

*Absorption spectra.* Curves taken at a point in a nucleolus (fig. 32) show the presence only of small concentrations of protein substances. A minimum is visible at 2,600 Å and there are no measurable quantities of nucleic acids. In addition, the unspecific

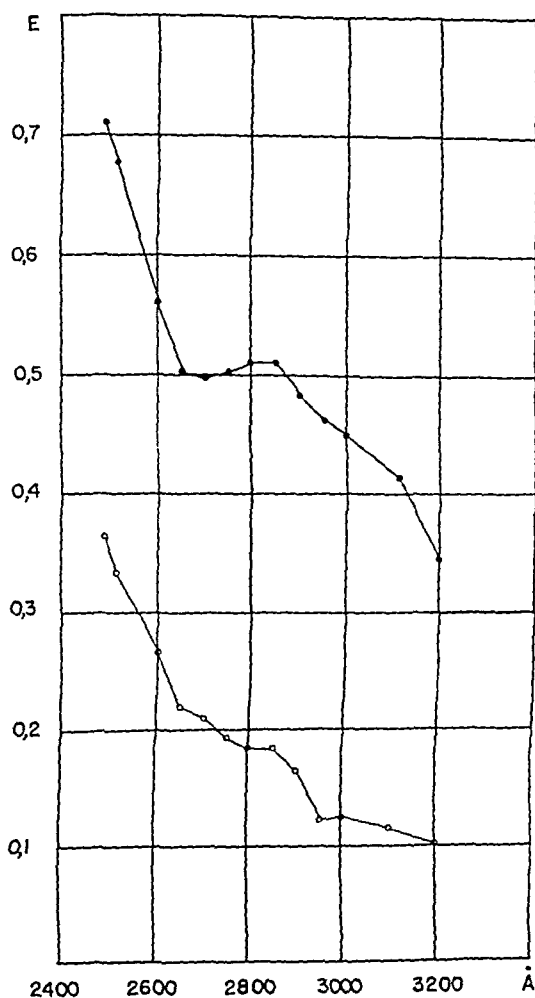


Fig. 32. Absorption spectra from points in the nucleolus and in the cytoplasm of the cell photographed in fig. 31.

losses of light are particularly great in these curves. All the curves from the cytoplasm only show the presence of an absorption at 2,800 Å. The remaining parts of the curves are conditioned by scattering of light in the specimen.

The microspectrographic analyses of this type of cell thus show that the intense ultraviolet absorption is mainly caused by scattering of light in the specimen. It was only possible to demonstrate the presence of small concentrations of protein substances.



Fig. 33. Two of the few remaining anterior horn cells from a rabbit after occlusion for 25 minutes and severe spastic paralysis for one month. The upper cell contains scarcely any absorbing substance at 2,570 Å. The cell below is intense absorbing and of the same appearance as the cell in fig. 31.

#### Cell changes after 30 days

Spastic paralysis developed rapidly in the hind legs, whose musculature became very atrophied in the course of the survival period. The survey specimen from that part of the spinal cord involved showed only a few remaining cells. The rest were neuronophago-



cytozed. Two types of cells were found. One was characterized by poor absorption capacity in the ultraviolet (fig. 33, upper photograph). The other type caused as a whole an intense absorption of the ultraviolet light (fig. 33, lower photograph). These cells were, however, smaller than the former and considerably smaller than corresponding cells from the controls.

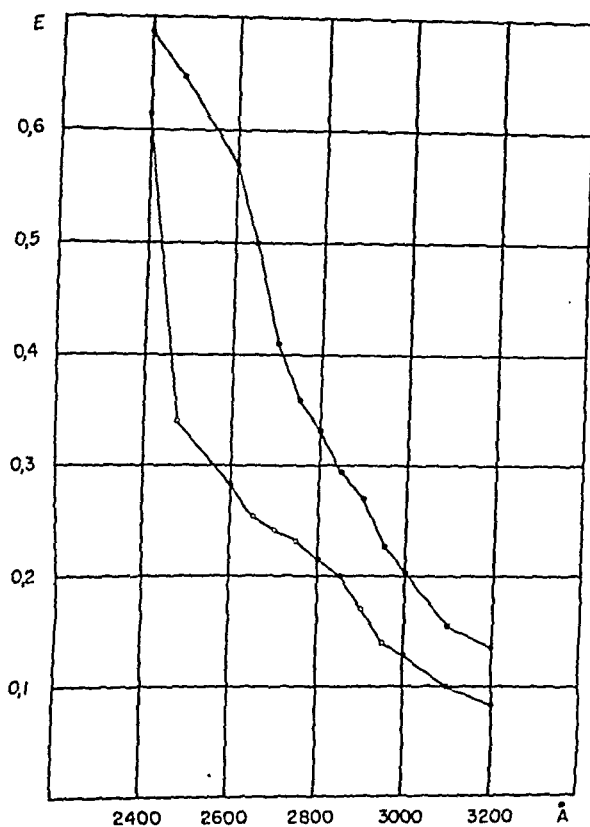


Fig. 34. Absorption spectra from points in the nucleolus and in the cytoplasm of the upper, faintly absorbing cell in fig. 33.

*Ultraviolet microscopy* (fig. 33, upper cell). The nuclear substance and the cytoplasm absorb poorly. The cytoplasmic substance is characterized by the presence of numerous small and large vacuoles.

*Absorption spectra.* The curve in fig. 34, taken at a point in the nucleolus, only shows the course of a light dispersion curve. The curve from the cytoplasm shows a weak absorption maximum at  $2,800 \text{ Å}$ , indicating small concentrations of protein substances. The losses of light due to scattering in the cell substance are considerable.

The other type of cell found in this animal is seen in the lower cell in fig. 33. In the ultraviolet photograph it is intensely absorbing. On absorption measurements, only typical curves for scattering of light were obtained. It was not possible to demonstrate any absorption maxima indicating the presence of nucleic acids or proteins in measureable quantities.

Thirty days after occlusion of the abdominal aorta for 25 minutes, the nerve cells thus lack nucleic acids and only contain protein substances in very small concentrations.

*Examples of Quantitative Data Obtained From the Motor Nerve Cells After Occlusion of the Abdominal Aorta for 25 Minutes.*

*Table VII. Severe Spastic Paralysis After Occlusion for 25 Minutes*

Animals killed	Cell detail	E 260	E 280	E corr 260	E corr 280	% Na
after 8 hrs (a)	cytoplasm	0.368	0.282	0.132	0.071	<0.8
	nucleolus	0.730	0.585	0.260	0.195	—
after 8 hrs (b)	cytoplasm	0.423	0.290	0.187	0.093	<0.2
	nucleolus	0.377	0.335	0.156	0.145	<0.2
after 48 hrs	cytoplasm	0.500	0.297	0.280	0.197	<0.2
	nucleolus	0.537	0.466	0.355	0.172	—
after 6 days	cytoplasm	0.263	0.180	0.103	0.080	—
	nucleolus	0.560	0.510	0.052	0.093	—
after 1 month	cytoplasm	0.282	0.217	0.146	0.102	—
	nucleolus	0.600	0.330	0.395	0.050	—

### Summary

Occlusion of the abdominal aorta for 25 minutes gives rise to changes in the motor nerve cells which very rapidly lead to an irreversible condition lethal for the cells and for function. The rapidly progressing chemical changes are characteristic. The results allow a detailed study of the endocellular mechanism during this process. The diagram in fig. 35 gives a survey of the results. Eight hours after the experiment there is a considerable decrease in the nucleoprotein content both of the nucleolar apparatus and of the cytoplasm. Six days and 30 days after the experiment only the structures remain of those cells that have not been neuronophagocytosed, so that they appear as hollow shells deprived of their contents. These cells

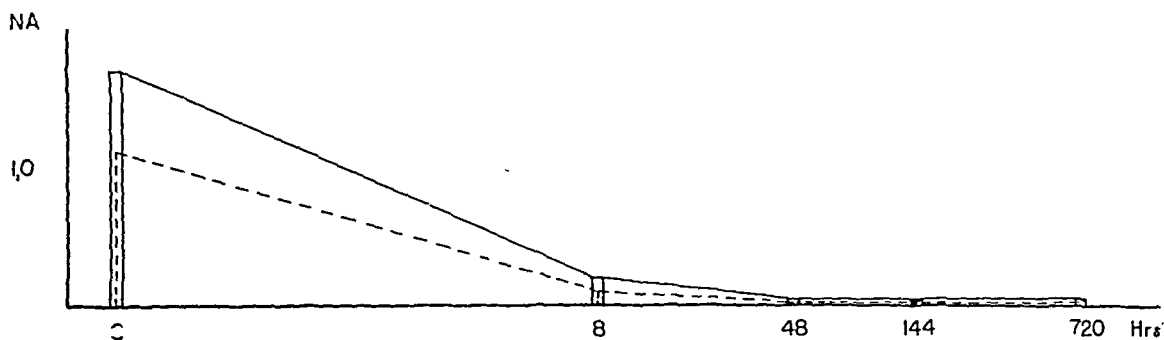


Fig. 35. Diagram showing the course of the quantitative changes in the nucleolus (*dotted lines*) and in the cytoplasm (*unbroken lines and white columns*) judged by absorption data obtained from the animals listed in table III. The abscissa gives the duration of the severe spastic paralysis in hours in logarithmic scale. After a duration of the paralysis for 8 hours there is a strong decrease in the nucleoprotein content of both the nucleolus and the cytoplasm. After 48—720 hours there is no measurable amount of nucleic acids in the remaining cells. Most cells are neuronophagocytosed.

correspond to those designated in the literature on cytology as pyknotic or strongly chromophil. Their intense stainability can not be conditioned by the presence of the acid groups in the nucleic acids but must be dependent on a physical adsorption of the dye groups.

From a neuro-physiological aspect, this experimental series is of less interest than the previous one. This series shows the mechanism of the chemical changes which lead to rapid destruction of the cell. The nucleoprotein content of the cytoplasm disappears rapidly. That of the nucleolus remains somewhat longer. The increase in the amount of chromocentre substance must be regarded as a desperate attempt at compensating activity on the part of the nucleoprotein-producing system.

#### Changes in the diameter of the nucleolus

Since the nucleolus occupies a key position in the system for the production of the cytoplasmic nucleoproteins, measurements of its diameter were made in the nerve cells of certain experimental animals in addition to the cytochemical analyses. The selection was made on the same principle as in the case of the cells for microspectrography, i. e.  $5\mu$  thick sections of anterior horn cells containing the main part of the nucleolus were taken. Three measurements were

made in the same cell and 100 cells in each experimental animal were measured. Measurement was carried out with a Zeiss ocular-micrometer, with a magnification of seven times. Optical system: objective apochromatic 90  $\times$ , numerical aperture 1.30. Table VIII gives the mean figures and the standard error of the mean both in the case of the controls and of specimens taken after a duration of the paralysis from two to 48 hours.

Table VIII

Animals killed	Range	Mean Value $\mu$	Standard Deviation	Standard Error of the Mean
Controls .....	4—8	5.96	$\pm 0.605$	0.061
after 4 hrs.				
Spastic paralysis for 2 hrs. ....	4—7	5.45	$\pm 0.57$	0.057
after 96 hrs.				
Spastic paralysis for 50 hrs. ...	4—7	5.43	$\pm 0.386$	0.039
after 8 hrs.				
Spastic paralysis for 8 hrs. ....	4—8	6.07	$\pm 0.70$	0.07
after 48 hrs.				
Spastic paralysis for 48 hrs. ...	4—7	5.70	$\pm 0.609$	0.061

A *t*-analysis according to FISCHER (1936) was made in order to establish the differences statistically. The calculations are shown in table VIII. The specimens were taken after paralysis had lasted for 2 and 50 hours respectively. Occlusion of the abdominal aorta lasted for 15 minutes.

According to the absorption data, a substantial decrease in the nucleotides in the nucleolus can be shown after two hours' paralysis. The figures after 50 hours are in excess of those for the controls.

As seen from the measurements, the change in the diameter of the nucleolus is statistically significant. The *t*-value after 2 hours' paralysis = 6.0.  $P = < 0.001$ . The *t*-value after 50 hours' paralysis = 7.4.  $P = < 0.001$ . This indicates that the quantitative changes in the nucleic acid content are true ones.

The calculation of the diameters of the nucleoli was also made in respect of the animals listed in table III after a duration of the paralysis of 8 and 48 hours respectively. The occlusion of the abdominal aorta lasted for 25 minutes.



Fig. 36. Two anterior horn cells from a rabbit after occlusion for 25 minutes and spastic paralysis for 48 hours. These cells demonstrate the ability of the chromocentre areas in the vicinity of the nucleolus to react by increasing their substance. See text.

The measurements show that no statistically significant enlargement in the diameter of the nucleolus has taken place 8 hours after the occlusion. The  $t$ -value = 1.2.  $P = > 0.2$ . It was not, however, possible to demonstrate any measurable amount of nucleic acids in the nucleoli after this paralysis.

## The reaction of the chromocentre areas

It was stressed earlier in this paper that the chromocentre areas in the nuclei of the nerve cells reacted strongly by increasing their substance. Since they contain protein substances and ribose nucleic acids, these areas are easily observable in the ultraviolet picture.

The phenomenon was interpreted as reflecting a compensating activity of the nucleoprotein-producing system against the ischaemic attack.

Since so obvious a reaction from these important nuclear areas seems remarkable, two examples with the most pronounced reaction are demonstrated. The cells in fig. 36 are taken from a rabbit after spastic paralysis for 48 hours. Several areas showing high absorption at  $2,750 \text{ \AA}$  are visible in the nucleus apart from the nucleolus. They belong to the chromocentre areas whose mass has thus substantially increased as compared with the control cells. The cytoplasm in these cells has a considerable nucleoprotein content. In fig. 36 they appear through their high absorption. We interpret this reaction as a defense mechanism thus showing a correlation between the chromocentre areas and the production of cytoplasmic nucleoproteins.

The observations agree with results previously obtained in analyses of the nerve cell nucleus under increased production of cytoplasmic nucleoproteins (HYDÉN 1943 b, HAMBÉRGER and HYDÉN 1948).

## Discussion: Significance of the Results Obtained

In a series of investigations with cytochemical methods, to which reference is made on p. 6, it was possible to demonstrate that a considerable consumption and production of ribose nucleic acids and protein substances takes place in the nerve cells. This occurs both in a relative condition of inactivity in the animal and in connexion with adequate stimulation and increased functional demands on the part of the neuron both in the motor and sensory nerve cells.

### The Significance of the Decrease in the Nucleoprotein Content of the Nerve Cells

A substantial decrease in the ribose nucleic acids in the cytoplasm of the nerve cells occurs, *inter alia*, in the following instances. On

trauma with impairment or cessation of neuronal function, the nucleoprotein production in the cytoplasm is temporarily inhibited or arrested. Examples of such an occurrence are the severing of the axon (HYDÉN 1943 b), when the nucleoprotein production is arrested for approximately 14 days, and acoustic trauma in the cochlear ganglion cells (HAMBERGER and HYDÉN 1945). The inhibition of the nucleoprotein production appears to take place through the effect on the nucleolus and the chromocentre areas.

With *irreversible damage*, the nerve cell loses its capacity for producing the nucleic acids and protein substances of the cytoplasm. An example is the infection by various neurotropic viruses (HYDÉN 1947). In such cases the mechanism of the attack can be studied and those parts of the nucleoprotein-producing system affected can be directly established. The virus can be regarded in general as a parasite on the nucleoprotein-producing system in the cell during its reproduction (CASPERSSON and HYDÉN 1945).

A disappearance of the cytoplasmic nucleoproteins need not, however, imply irreversible damage. This is shown by our results reported in another type of neuronal injury. This can be said to result both in a cessation of function, i. e. paralysis, and in the presence of a dysfunction, i. e. the spastic condition — muscular hypertonus.

The results of occlusion of the abdominal aorta for 15 minutes in series I and II are intrinsically the same, i. e. slight or moderate spastic paralysis, reversible in some cases.

A collocation of the results shows that considerable quantitative chemical changes take place whereas there are no changes in the symptomatic picture of the paralysis. With its onset, a substantial decrease in the cytoplasm content of nucleoproteins occurs. There is an inhibition of the nucleoprotein-producing system in the nerve cells, which can be said to lie fallow for a time. *The results indicate that the maintenance of a constant production of nucleoproteins is a condition for physiological neuronal activity.*

The amount of nucleoproteins in the cytoplasm increases during a subsequent phase of restitution which, in our experiments, precedes functional restitution.

Under these conditions, the demands on the capacity of the nucleoprotein-producing system are considerable. It has been possible to follow quantitatively the changes in the nucleoprotein content in the most important organella in this system, i. e. the nucleolus. The

nucleoprotein content is unaltered at the onset of paralysis and during the phase of restitution. The experiments with binding of dyes as well as the absorption spectra show a high nucleoprotein content in the nucleolus and a high content of basic groups. This is interpreted as a nucleolar apparatus in a phase of intense stimulation.

The chromocentre areas in the nucleus constitute that part of the nucleoprotein-producing system which, from a point of view of development, is earlier than the actual nucleolar mass. During spastic paralysis, particularly in the phase of restitution, the substance in the chromocentre areas increases considerably. This is interpreted as a compensating activity. *The results show that a decrease in the cytoplasmic nucleoproteins does not indicate irreversible damage as long as the nucleoprotein content of the nucleolus is satisfactory.*

### **The Cytochemical Correlate of "Spasticity"**

"Spasticity" in the sense of muscular hypertonus without the development of paralysis appears to have a distinct cytochemical correlate. The nucleoproteins in the cytoplasm are in agglomerations without any distinct borderlines, thus giving a characteristic appearance to the cell in the ultraviolet. A similar ultraviolet picture and quantitatively the same amount were found in sensory nerve cells after intense adequate stimulation (HAMBERGER and HYDÉN 1948), after stimulation of the spinal ganglion cells (HYDÉN 1943 b) and in the nerve cells of Deiters' nucleus after repeated adequate stimulation resulting in signs of vestibular fatigue (HAMBERGER and HYDÉN 1948).

*The results indicate a condition of strong stimulation or irritation in the motor anterior horn cells during the dysfunction termed spasticity.*

### **The Course in Destruction of the Neuron**

Pronounced spastic paralysis developed in the hind legs of the experimental animals after 25 minutes' occlusion of the abdominal aorta. The cytological changes were severe and resulted in death of the cell and simultaneous cessation of function. Our results permit a study of the mechanism in the irreversible intracellular processes in this injury. A disappearance of the nucleic acids and protein substances both in the cytoplasm and in the nucleolus is characteristic. After such severe chemical changes in the nucleolus, restitution does



not appear possible. *The results of our findings, assembled in the diagram (fig. 35) appear to be suitable for use as comparative values in a prognostic assessment of the condition of the nerve cells.*

EINARSSON has studied the nerve cells under various functional conditions in extensive investigations. Mention will only be made here of his work on vitamin E deficiency (EINARSSON and RINGSTEDT 1938) and of experimentally induced neuromuscular symptoms in animals (EINARSSON 1933, 1945, EINARSSON and LORENTZEN 1946). He describes different cytological changes in the nerve cells as typical of varying conditions of activity.

EINARSSON uses staining with galloxyaniline-potassium chromic sulphate. Since nucleic acids are strongly tetrabasic acids, it is probable that the intense stainability in the nerve cells with this method is due to their ribose nucleic acid content.

EINARSSON's theory that moderate chromophilia in the nerve cells reflects generally increased activity, and chromophilia lengthy activity is entirely in agreement with the opinions of earlier workers and with our own results.

In EINARSSON's opinion, nerve cells showing strong chromophilia contain large concentrations of nucleic acids and protein substances and indicate depressed activity. There could then be no consumption of the nuclear and cytoplasmic nucleoproteins since no discharge from the cell takes place.

We studied nerve cells with this appearance on staining (v. pp. 48—51) but found that they contained no measurable quantities of nucleic acids. On the other hand, the physical condition of the cell substance is altered and there are considerable light losses due to scattering of light.

### General Summary

Spastic paralysis is induced in rabbits by means of bloodless occlusion of the abdominal aorta according to HÄGGQVIST's method.

The motor anterior horn cells in the antero-lateral group of the spinal cord in the area affected are studied with microspectrographical and other cytochemical methods at varying times after the experiments. The cell changes are assessed after a duration of the paralysis varying between 15 minutes and 30 days.

It is possible to correlate the changes in the nerve cells with the course of the functional changes. Slight or moderate paralysis

developed in two series after occlusion for 15 minutes. During the residual paralysis extensive chemical changes are reflected in the nerve cells. The amount of the ribose nucleic acids and proteins in the cytoplasm decreases with its onset. A restitution phase, whose mechanism is studied, then sets in.

A study is made of the quantitative chemical changes in the nucleolus. The chromocentre areas constitute another important part of the nucleoprotein-producing system in the nerve cells. Its nuclear parts react intensely in a compensatory direction.

Muscular hypertonus appears to have a characteristic cytochemical correlate in the motor cells.

Severe spastic paralysis develops after occlusion of the abdominal aorta for 25 minutes. The majority of the anterior horn cells then undergo severe changes and are neuronophagocytosed. The nucleic acids and proteins in both the nucleolar apparatus and the cytoplasm disappear at the onset of paralysis and are not replaced by new production.

This nucleolar change is a severe prognostic sign that can be used to assess the condition of vitality in the nerve cells.

Remaining nerve cells contained no measurable quantities of nucleic acids.

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